Interrelations of *Ips avulsus* (Eichh.) and Associated Fungi

By

RICHARD JAMES GOUGER

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
1971

ACKNOWLEDGEMENTS

The writer wishes to acknowledge his indebtedness to the chairman of his Supervisory Committee, Dr. R. C. Wilkinson, for his constant guidance, patience, assistance, and advice throughout the course of this study. Special thanks are due to Dr. James W. Kimbrough, particularly with respect to the fungi isolated in this study. The writer also wishes to express his appreciation to Drs. D. H. Habeck, L. A. Hetrick and F. W. Zettler for their criticism on the preparation of the manuscript. Thanks are also extended to the above, for serving on the writer's Supervisory Committee. Sincere gratitude is extended to Mr. W. J. Coleman for assistance in both the field and laboratory phases of this study.

Thanks are accorded to Mr. D. P. Jouvenaz with respect to the bacteria associated with *Ips avulsus*, and to Dr. H. J. Phaff, Department of Food Science, University of California, Davis, for identification of the yeast fungi.

The generous assistance of Mr. James B. Gouger in the preparation of the figures in the manuscript and Mrs. Rosemary Rumbaugh in preparation of certain histological sections is gratefully acknowledged.

This study was partially supported by funds made available by the Southern Forest Disease and Insect Research Council, Southern Pulpwood Conservation Association.

A special kind of appreciation is due my family, for their understanding during the course of this work.

TABLE OF CONTENTS

TABLE OF CONTENTS—*Continued*

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS—*Continued*

LIST OF ILLUSTRATIONS—*Continued*

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

INTERRELATIONS OF *IPS AVULSUS* (EICHH.) AND ASSOCIATED FUNGI

By

Richard James Gouger

August, 1971

Chairman: Dr. R. C. Wilkinson
Major Department: Entomology

Ips avulsus (Eichh.) is closely associated with certain fungi, and these fungi are introduced into trees by this beetle. Histological evidence and examination of whole mouthparts suggested that transmission of the fungi from one generation of *Ips avulsus* to another is accomplished entirely by means of spores and yeast cells either adhering to the external parts of the insect or passing through the intestinal tract.

Aspects of the life cycle of *Ips avulsus* are discussed with special elaboration on reproductive and feeding behavior and relative possibilities for fungus transmission.

Three ectosymbiotic fungi were found to be consistently associated with *I. avulsus* in Florida. They included *Ceratocystis ips* (Rumbold) C. Moreau, the well-

known bluestain fungus, and two species of yeasts, *Hansenula holstii* Wickerham and *Pichia pini* (Holst) Phaff. Another fungus, tentatively identified as an undescribed species of *Ambrosiella* Brader ex V. Arx and Hennebert, was frequently associated with *I. avulsus* during the winter and spring.

Interactions of paired fungi isolated in this study showed that none of the fungi in any combination were stimulated to increase their growth rate. It was hypothesized that an inter-dependency for nutrients, especially organic nitrogen, was responsible for the reduced growth rates and sporulation.

Some new and modified techniques were described as an aid to further studies of the bark beetle-fungi complex. These include methods for observing bark beetle behavior and methods to evaluate fungus interactions. It was suggested that one or both of the yeasts and/or bacteria isolated in this study were factors contributing to more rapid development and larger wild *I. avulsus* broods, as compared to aseptic broods reared in the absence or presence of *Ceratocystis ips*.

CHAPTER I

INTRODUCTION

Interactions of the bark beetle, *Ips avulsus* (Eichh.), with its various fungal associates, and typical slash pine, *Pinus elliottii* var. *elliottii* Engelm., present a study in symbiotic relationships. DeBary (1879) and Leach (1940) defined symbiosis as the living together of dissimilar organisms, regardless of the results of the association. One common symbiotic relationship in bark beetles is that described by Graham (1967) as mutualism, in which mutual advantages accrue from a close association between individuals of different species. This would exclude associations in which two species merely coexist or compete, or in which benefits are provided to only one of the species. Mutualism does not prescribe obligatory dependency of one partner on the other, although such dependency is not uncommon. Mutualism would include associations in which a fungus becomes the food source or modifies the tree as a food medium for the insect, as well as those associations which enable an insect to successfully persist in and successfully colonize a host tree.

To date, three categories of ectosymbiotic fungi have been associated with bark beetles in the genus *Ips* DeGeer. They are the ambrosia fungi, wood-staining fungi,

and yeasts. Ectosymbiosis pertains to symbiosis in which such fungi can grow and reproduce independently from their insect vectors; the insects are benefited by the fungi becoming all or part of their food supply or by environmental conditions becoming favorable to the insect. The term "fungi" includes not only the nonchlorophyllous plants possessing mycelium, but also the nonfilamentous yeasts.

Much of the literature on symbiosis between scolytid beetles and ambrosia fungi has been reviewed by Francke-Grosmann (1967). The research covered did not give proof that these fungi produced specific nutrients essential to the beetles; however, recent work by Chu et al. (1970) on the effects of a mutualistic ambrosia fungus, *Fusarium solani* (Sacc.), upon the growth and reproduction of *Xyleborus ferrugineus* (F.) showed that this fungus supplied a source of ergosterol which was essential for pupation in this ambrosia beetle. Ergosterol is the principal sterol of fungi, as discussed by these authors.

Wood-staining fungi-beetle associations have been reviewed by Mathiesen-Kaarik (1953), and Francke-Grosmann (1963, 1967). A general conclusion is that wood-staining organisms transmitted into a tree by bark beetles during attack or preoviposition feeding render the tree a more suitable breeding medium for the beetles. It is not entirely

clear in what specific ways the beetles are benefited, however.

The occurrence of yeasts associated with bark beetles has been reviewed by Mrak and Phaff (1948), Shifrine and Phaff (1956), and Callaham and Shifrine (1960). Earlier workers generally considered yeasts to be of little benefit to associated insects, but at least three functions have been suggested by more recent workers. Webb (1945) and Rumbold (1931) proposed that certain yeasts may accelerate the growth of ambrosia and wood-staining fungi. Wilson (1959) suggested that certain yeasts render the substrate a more suitable medium for ambrosia fungi by breaking down tannins into fungal nutrients. Hogan (1948) and Farmer (1965) attributed a direct nutritional role to those yeasts which serve as food for an insect.

One important test concerning the association of ectosymbiotic fungi with bark beetles is the demonstration of a close relationship between insect and fungus. Francke-Grosmann (1956a) devised a method, which she called "fractional sterilization," which helps to clarify such a relationship. This method depends upon exposing the insects to three cycles of alternately moist and dry conditions. Chance external contaminants germinate during the moist phase and die during the dry phase, while the symbiotic

fungi often are unaffected. This technique favors isolation of fungi adapted for beetle transmission. Spores of such fungi commonly secrete a mucous coat which prevents digestion during passage through the insect gut. The spores apparently resist fractional sterilization because of this coat and/or because they are in protected situations within the insect.

To demonstrate the type of association among bark beetles and their fungal associates, it is desirable that "control" beetles be reared free of their symbionts. Holst (1937) in the United States and Grosmann (1930) in Germany artificially reared small numbers of certain bark beetles from egg to adult free of their bluestain fungal associates and concluded that the fungi were not essential for development within a single generation of the beetles. Hetrick (1949) observed an infestation of the southern pine beetle, *Dendroctonus frontalis* Zimm., in which the trees showed no signs of bluestaining. Since beetle brood development appeared to be normal, he concluded that bluestain organisms were not essential for successful attack by this beetle.

Yearian (1966) reviewed bluestain fungus-*Ips* bark beetle relationships and investigated this association in Florida. He found that the bluestain fungus, *Ceratocystis ips* (Rumbold) C. Moreau, was consistently associated with

and transmitted by *Ips avulsus* (Eichh.), *Ips calligraphus* (Germ.), and *Ips grandicollis* (Eichh.) infesting *Pinus* species in Florida. No significant differences were found in egg gallery length, brood size, brood composition, pupal weight, or fecundity of aseptically reared, bluestain-free and bluestain-inoculated *Ips avulsus* populations. Similar results were obtained with *Ips calligraphus* and *grandicollis* populations in most tests. Bluestain-free populations of the three *Ips* species were successfully reared through 3-4 successive generations in fungus-free pine logs. *Ips* species oviposition was inhibited when pine logs were inoculated with *C. ips* prior to introduction of the adult beetles, otherwise the bluestain fungus did not significantly affect beetle development in pine logs.

Wild *Ips avulsus* populations produced significantly longer egg galleries, laid more eggs, and produced larger, heavier broods that matured faster than did bluestain-free or bluestain-inoculated populations, a result attributed to the elimination of one or more associated microorganisms (other than *C. ips*) by aseptic treatments.

This study is partially a continuation of Yearian's investigations with emphasis placed on the following:

1. mechanism(s) of fungus transport in *Ips avulsus;*
2. reproductive and feeding behavior of *Ips avulsus* and related possibilities for fungus transmission;

3. isolation and identification of fungi associated with *Ips avulsus;* and

4. studies of the interactions of the fungi associated with *Ips avulsus*.

The results of these investigations are reported herein.

CHAPTER II

FUNGUS TRANSPORT IN *IPS AVULSUS*

Background

Specialized structures or repositories for carrying symbiotic fungi have been described in a number of bark beetles by various workers. Francke-Grosmann (1963) described in *Ips acuminatus* (Gyll.) the presence of such an organ in the females situated behind the mandibles and containing fungal spores. Since *Ips avulsus* has been shown to be closely associated with several species of fungi (Rumbold 1931, Yearian 1966), it seemed reasonable to suspect that this bark beetle would possess some type of specialized fungus-carrying organ. The purpose of this study was to determine the presence or absence of such structures in *Ips avulsus*.

Paired fungus-containing structures of ectodermal origin in beetles initially were termed "mycangia" (Batra 1963); Giese (1966) applied the term "mycetangia" to paired organs in which microsymbionts are stored and from which they are transmitted. Other workers have found that some fungus repositories are not paired (Farris and Funk 1965), and have proposed the use of "mycangium" as any specialized structure of a beetle in which fungus is stored. In view

of this research, my use of "mycangium" will be in the latter sense. A review of the occurrence of organs found in different ambrosia beetles has been given by Francke-Grosmann (1967).

Most of the work on mycangia in bark beetles has been conducted by Nunberg (1951); Francke-Grosmann (1956a, 1956b, 1958, 1963, 1966); Fernando (1960), Lhoste and Roche (1961); Francke-Grosmann and Schedl (1960); Baker (1963); Batra (1963); Finnegan (1963); Farris (1963); Farris and Funk (1965); Abrahamson and Norris (1966); Lowe, et al. (1967); and Schneider and Rudinsky (1969).

Although mycangia are situated in various parts of the head or thorax of adult beetles, they have several common features. Francke-Grosmann (1963) recognized 2 types of organs: Mycangial cavities developed as modifications of already existing organs, for example, the scutellar fold in *Anisandrus dispar* (F.) (preformed mycangia), and independent structures (independent mycangia) as found in *Trypodendron* spp. All are invaginations of the epidermis and have a cuticular lining. Various structures, such as hairs or ridges, close the openings. The stored fungi are expelled indirectly in different species by the movement of thoracic muscles, legs, wings, or mandibles.

In most species, mycangia are present in females (Farris and Funk 1965; Fernando 1960; Francke-Grosmann 1956a,1963, 1965). However, in some species these organs are also found in males (Farris 1963; Finnegan 1963). One report by Abrahamson and Norris (1966) describes oral mycangia from both sexes of *Xyloterinus politus* (Say) and prothoracic mycangia only in females.

In addition to the presence of mycangia of ectodermal origin in many insects, there are diverticula or gastric caeca of endodermal origin located on the mesenteron of the alimentary canal of certain Hemiptera which serve as repositories for microsymbionts (Leach 1940). Thomas (1967) described gastric caeca on the midgut of the alimentary canal of the larvae and adults of a number of bark beetles including *Ips avulsus*, but he did not discuss their function. Since gastric caeca have been reported as repositories for microsymbionts in other insects, these structures were examined in *Ips avulsus* to determine whether microsymbionts were present.

Stridulating organs on the head and pronotum of female *Ips avulsus* were noted by Wilkinson (1962). The structure of these sound-producing organs in *Ips calligraphus* females was illustrated by Wilkinson et al. (1967). They found that the plectrum (fixed stridulating plate) located

on the underside of the pronotum was associated with a cavity within the pronotum. Since this structure is also found in female *Ips avulsus*, it was suggested that the cavity might function as a mycangium (Wilkinson, personal communication). Therefore, the stridulating organ cavity in *Ips avulsus* was examined to determine whether microsymbionts were present.

Methods

Both sexes of *Ips avulsus* adults were collected during June, 1967 as they emerged from typical slash pine near Gainesville, Florida. Ten beetles of each sex were prepared for serial sectioning and staining to determine the presence or absence of mycangia. Specimens were fixed for 48 hours in Carnoy's fluid (Appendix). The beetles were washed in two changes of 95% ethyl alcohol after fixation and immediately embedded in Paraplast embedding medium (melting point 56-57°C; Fisher Scientific Company). Several transverse and sagittal sections were cut at 8-10μ. The ribbons were spread on a water bath at 43°C and affixed to slides with Haupt's adhesive (Appendix).

After sectioning, insect tissues and microorganisms were differentiated by a modified Gram-Weigert procedure (Appendix) (Mallory and Wright 1924) which stains yeasts, fungi, and Gram-positive bacteria blue and insect tissues red.

Two methods were used to examine the gastric caeca for the presence of microsymbionts. The first method consisted of removing the entire alimentary canal using the method described by Thomas (1967). Each adult bark beetle was immersed in a small dish of 70% ethanol and grasped with two pairs of forceps, one about the thorax and the other about the anal region. By tearing the integument at the anus, the entire gut up to the proventriculus was extracted from the body with all caeca intact. After dissection, the caeca were stained by the modified Gram-Weigert procedure and examined under a compound microscope for the presence of microorganisms. Ultra-thin sections of the gastric caeca were also prepared and examined with an electron microscope for microsymbionts. The procedure used was one described by Dr. H. C. Aldrich of the University of Florida, Department of Botany (unpublished data) (Appendix). Whole mounts of mouthparts were prepared and examined for the presence of mycangia under a compound microscope.

Results and Discussion

Although it has been clearly demonstrated that several fungi are associated with *Ips avulsus*, and that *C. ips* is disseminated and introduced into trees or

logs and logging debris by these beetles (Yearian 1966), no specialized structures for carrying fungi in this insect were found in this study.

Examination of serial sections of adult males and females (Figures 1-4, Plate I) showed no concentrations of microorganisms in the various structures examined. Such concentrations are characteristic of mycangia.

The cavity associated with the plectrum on the underside of the pronotum in female *Ips avulsus* did not contain microorganisms (Figure 5, Plate I).

Histological studies of the beetles revealed the universal presence of fungal spores, both externally, adhering to the exoskeleton (not shown in plates), and internally, in the digestive tract (Figures 7-8, Plate I). No special symbiont-containing structures were found adjacent to the vagina as reported in two scolytid subfamilies (Aslam 1961).

A histological study of the two types of gastric caeca described by Thomas (1967) in *Ips avulsus* yielded no evidence of fungal spores in these structures (Figures 1-3, Plate II).

In summary, no histological evidence was found of the existence of mycangia in adult *Ips avulsus* males and females.

Histological evidence and examination of whole mouthparts suggested that transmission of the fungi from one generation of *Ips avulsus* to another is accomplished entirely by means of spores and yeast cells either adhering to the external parts of the insect or passing through the intestinal tract, as found by Leach et al. (1934) in 2 other species. A characteristic which favors endozoic transfer is the investment of spores by a mucilaginous capsular material which apparently protects them from being digested in the gut (Francke-Grosmann 1963). At least 3 of the fungi isolated in this study possess this characteristic. *C. ips* ascospores are always imbedded in a gelatinous sheath (Moreau 1952). Both yeasts associated with *I. avulsus* (Chapter IV) also produce a phosphorylated mannan sheath (Wickerham and Burton 1961). Slodki (1962, 1963) has discussed the chemistry of this material. Basically it is an adhesive polymer with properties similar to polysaccharide gums. It is reported to be quite refractory to enzyme attack, which would probably make the yeast species possessing it less susceptible to the action of digestive juices.

The absence of mycangia in *Ips avulsus* and the investment of spores of the associated fungi by capsular sheaths suggest that the fungi are adapted for beetle transmission rather than the reverse case.

PLATE I

Figures 1-4. Sagittal sections of a female *Ips avulsus* stained with a modified Gram-Weigert stain. 1, section through trochanter. T, trochanter; H, head. 2, section through coxae. C, coxa, H, head. 3, section through mandible. M, mandible; H, head. 4, median sagittal section. H, head; E, esophagus. (Approximately ×20)

Figure 5. Sagittal section through pronotum showing cavity in plectrum. P, plectrum; C, cavity. Anterior portion of pronotum to left. (Approximately ×230)

Figure 6. Sagittal section through pars stridens. P, pars stridens; E, esophagus. (Approximately ×40)

Figure 7. Median sagittal section through abdomen showing mass of bark fibre, fungal spores and starch grains in midgut and hindgut. The various materials are not clearly differentiated in this photograph. MG, midgut; HG, hindgut. (Approximately ×30) (Anterior direction to right)

Figure 8. Median sagittal section through ventricular area. P, proventriculus; MG, midgut. (Approximately ×30)

(All photos by author)

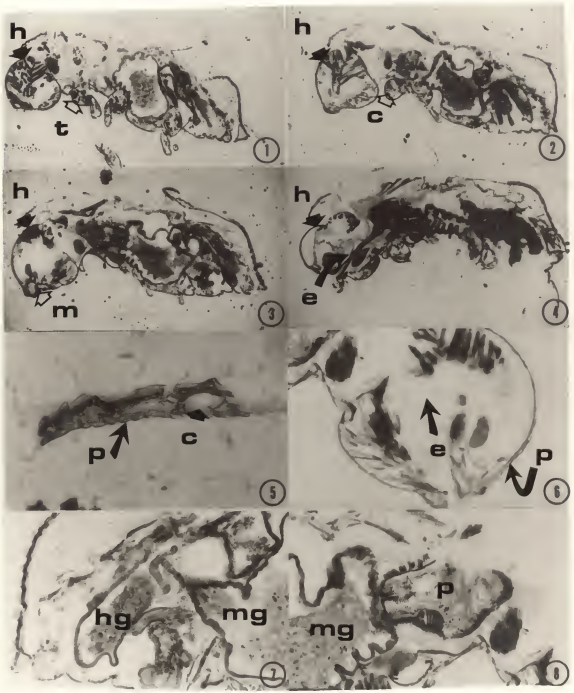
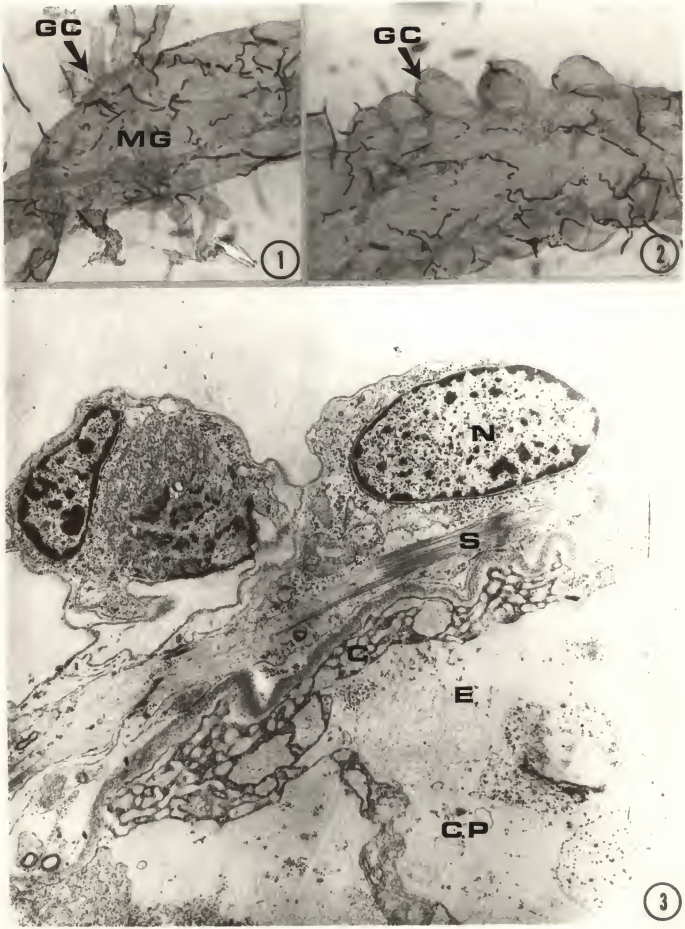

h
t
1
h
c
2
h
m
3
h
e
4
p
c
5
e
p
6
hg
mg
7
p
mg
8

PLATE II

Figure 1. Whole mount of elongate gastric caeca on midgut of *Ips avulsus*. The dark lines in the photograph are tracheae. GC, gastric caecum; MG, midgut.

Figure 2. Whole mount of globular gastric caeca. GC, gastric caecum. (Photographed ×40 and enlarged) (Photo by author)

Figure 3. Electron micrograph of cross section of globular gastric caecum showing structure of wall. N, nucleus of striated muscle; S, striated muscle fibre; E, portion of epithelial cell; C, cytoplasmic membrane; CP, cytoplasm. (12,600×) (Photo by R. Rumbaugh) No concentrations of microorganisms were found in these caeca. (Photographed ×40 and enlarged) (Photo by author)


GC
MG
1
GC
2
N
S
C
E
CP
3

CHAPTER III

REPRODUCTIVE AND FEEDING BEHAVIOR OF *IPS AVULSUS* AND RELATED POSSIBILITIES FOR FUNGUS TRANSMISSION

Background

During studies to determine the interrelations of *I. avulsus* and its associated fungi, it became apparent that a knowledge of the behavior of this beetle during gallery construction, mating and oviposition would be valuable since it might illustrate methods by which the various fungi are transmitted between beetles of the same or successive generations.

The behavior of several scolytids during gallery construction, mating and oviposition has been recorded. Chamberlin (1939) described mating in *Scolytus rugulosus* (Ratzeburg), Goeden and Norris (1965) discussed gallery construction, mating, oviposition, and post-oviposition behavior in *Scolytus quadrispinosus* Say, and Wallace (1939) and Gouger (1966) discussed mating behavior in *Scolytus multistriatus* (Marsham). Reid (1958) recorded gallery construction, mating, and oviposition in *Dendroctonus ponderosae* Hopkins, Francke-Grosmann (1959) described mating in *D. micans* (kugelann) and Yu and Tsao (1967) in *D. frontalis* Zimmerman. Blackman (1915) has described mating behavior in *Pityogenes hopkinsi* Swaine.

Stridulation behavior and its role in *Ips calligraphus* reproduction has been reported by Wilkinson et al. (1967). Wilkinson and Gouger (unpublished data) also observed and recorded mating and oviposition behavior in *I. calligraphus*. There is no published information on the habits of *I. avulsus* during gallery construction, mating and oviposition, however.

Methods

The reproductive behavior of *Ips avulsus* was studied in observational plates constructed of the following materials: a 10 × 14 × 3/8-inch glass plate which was placed on a flat surface; a 15 × 24 × .5=cm. sheet of phloem from the inner bark of *Pinus elliottii* Engelm. var. *elliottii* which was placed on the glass plate; a glass plate (10 × 14 × 3/8 inch, with a 6-mm. hole in the center) which was placed over the phloem sheet; then no. 10 binder clips which held the apparatus together; a 6 mm. plug of outer bark which was inserted in the hole in the glass; and nonabsorbent cotton which was packed around the edges of the phloem sheet to prevent desiccation. All plates were held at 30°C throughout the study. This method is a modification of a "sandwich" technique described by Hopping (1961) for rearing *Ips* spp. The term "phloem" used in this text refers to the inner portions of the bark, just outside the cambium.

All test beetles were collected as callow adults from or near their pupal cells in inner pine bark. It was assumed that they had not mated prior to the experiment because young adults were never observed to mate in the sandwich units in which they had been reared as an F1 generation.

To initiate each test, one male was placed on the outer bark plug and a 10-mm. cover slip was placed over the hole. The outer bark plug was used to simulate field conditions in which the male beetle tunnels through the corky outer bark to reach the phloem underneath. Males that did not become established by constructing nuptial chambers were replaced by other male beetles, as required. Twelve hours after the male was introduced and established, a virgin female was placed on the bark plug.

Gallery construction, mating and oviposition were quantitated with a hand rule and stopwatch, and field observations supplemented these studies. Observations were carried out for the entire mating period per pair (until each female had isolated herself from the associated male by plugging the egg gallery with frass).

Results and Discussion

Behavior Associated with Initiation of Beetle Attack

The location of the attack sites in pine logs in the field corresponded to those of Blackman (1915) for

S. quadrispinosus Say. He reported that the entrance holes were begun in the thinner portions of the bark, in longitudinal cracks, or under the bark scales of hickory trees (*Carya spp.*).

The male *I. avulsus* rotated about his longitudinal axis while constructing an entrance tunnel. Most frass was passed between the mandibles, maxillae and prothoracic legs and pushed to the rear with the meso- and metathoracic legs. When the male beetle reached moist phloem tissue, this material was excised and compressed between the mandibles and maxillae. The compressed phloem tissue was then expelled from the mouth and pushed to the rear as described above. Liquid material and starch grains were squeezed out of the phloem tissue and ingested, corresponding to the large number of starch grains and small amounts of phloem tissue observed in serial sections and whole mounts of *I. avulsus* digestive tracts taken from attacking beetles (Plate I, Figures 1-4).

Although it has been demonstrated that males of certain *Ips* bark beetles normally initiate attacks on host material and attract other adults of both sexes (Anderson 1948; Wood and Vité 1961; Wilkinson 1964), there is no published evidence that *I. avulsus* males do likewise. In this study, observations suggested that *I. avulsus* males

did emit an attractive substance several hours after attack was initiated. All females placed at the male entrance hole in pine bark became visibly excited upon contacting inner bark frass freshly extruded from the nuptial chamber, and burrowed into this frass until they were at the entrance to the tunnel leading into the nuptial chamber.

Mating Behavior

The behavior of mature males and females at the nuptial chamber entryway corresponded to that described by Clemens (1916) in *Ips pini* (Say), Wilkinson et al. (1967) in *I. calligraphus*, and by Barr (personal communication) in *I. confusus (paraconfusus)*. The males blocked the entry to the nuptial chamber by backing into the entry and bringing the scooped-out posterior elytral declivity even with the entrance. The female beetle pushed vigorously against the male declivity when attempting to enter the nuptial chamber and stridulated only when contacting the male. The pushing stridulating behavior of the female was repeated until the pair reached the nuptial chamber (Plate III, Figure 2; Plate IV, Figure A). The female occasionally bit the margins of the male elytra while forcing her way into the gallery. Immediately after entering the nuptial chamber, the female started excavation (Plate IV, Figure B). At this time, the

males assisted the females by removing frass (Plate IV, Figure C). The females did not stridulate after initiating egg galleries.

Copulation was first observed only after females had constructed 2.5 - 3.0 mm. (or about one body length) of an egg gallery (Plate III, Figure 3; Plate IV, Figure D). To copulate, the male first butted the female's abdomen. This behavior apparently stimulated the female to receive the male. After stimulation was effected, copulation occurred at an angle of about 100° (Plate III, Figure 3; Plate IV, Figure D). Copulation was of short duration and an average of 35 seconds (range 22-45 seconds) was spent in copula by 10 beetle pairs at 30°C. Each pair copulated an average of 3 times during the entire mating period. Copulation occurred at 10-minute intervals during egg gallery construction. Repeated copulations apparently are common in *Ips* spp. and *I.calligraphus* pairs copulated before the deposition of each egg (Wilkinson and Gouger, unpublished data).

Oviposition Behavior

After excavation of the nuptial chamber the female preceded the male and began to construct an egg gallery (Plate IV, Figure C). This was usually constructed parallel with the grain of the phloem. Several galleries were noted

at an angle of 45° with the grain, although the absence of xylem (wood grain) in the sandwiches might account for this behavior.

Oviposition in phloem sheets corresponded with the description of egg laying by Reid (1958) for the mountain pine beetle, *Dendroctonus ponderosae* Hopk. The female first cut out an egg niche on the side of the gallery wall and the phloem material excised from the egg niche was packed in a plug at the end of the gallery (Plate III, Figure 4; Plate IV, Figure E). She then backed out of the gallery (Plate V, Figure F), turned around in the nuptial chamber (Plate V, Figure G), proceeded backward to the niche (Plate V, Figure H), and oviposited in the egg niche (Plate III, Figure 5; Plate V, Figure I). Finally, she again reversed her position in the gallery (Plate VI, Figures J, K, L) and packed the phloem from the phloem plug around the egg with her mouthparts (Plate III, Figure 6; Plate VI, Figure M).

The eggs were deposited at irregular intervals in niches cut in both sides of the gallery. When *I. avulsus* females constructed galleries side by side, most of the eggs were deposited in the outer (farthest removed) sides of their respective galleries. These observations correspond to those of Yearian (1966).

Egg gallery construction and oviposition in pine logs were measured by Yearian (1966). Most egg gallery construction and oviposition was completed 10 days after introduction of the females and most of the activity took place during the first 5 days. *I. avulsus* had a mean egg gallery length of 13.5 ± 1.4 cm., a mean spacing between eggs of 0.59 cm., and a mean brood size of 20.7 ± 1.6 eggs. *I. avulsus* females abandoned their galleries between 18 and 20 days after introduction into logs.

Transmission Possibilities

The primary objective of this study was to observe ways by which various fungi might be transmitted from one beetle or generation to another. These observations suggested several ways in which the various associated fungi might be introduced: (1) The fungi associated with *I. avulsus* have mucilaginous spores which readily adhere to the beetles exoskeleton. Many spores are probably brushed off when the beetles construct their galleries, as suggested for *I. grandicollis* by Leach et al. (1934), (2) A large portion of the host material ingested by the beetles was liquid and gelatinous feces were commonly deposited in the nuptial chamber. Since large quantities of viable spores were present in the hind gut of adult beetles, it is suggested that a large number of spores were transported and

inoculated into the galleries via contamination of beetles bodies with gelatinous fecal material, (3) The female beetle formed the phloem plug from mascerated phloem plus an adhesive-like material. The source of this material was not determined. However, if it were regurgitated from the digestive tract, it would probably contain large numbers of fungal spores.

In summary, several ways in which fungi may be introduced by *I. avulsus* are: (1) spores and cells are brushed off when the beetles construct their galleries; (2) spores and cells are deposited into the galleries via gelatinous fecal material; (3) spores and cells are inoculated in phloem plugs.

PLATE III

Figure 1. Male bark beetle constructing nuptial chamber: NC, nuptial chamber; ET, entrance tunnel.

Figure 2. Female following male into the nuptial chamber.

Figure 3. Copulation in nuptial chamber and beginning of egg gallery: EG, egg gallery.

Figure 4. Female constructing egg niche: EN, egg niche; FP, phloem plug.

Figure 5. Female ovipositing in egg niche.

Figure 6. Female packing phloem from the egg niche around the egg.

(All photos by author; a much larger species, *Ips calligraphus*, was used to illustrate the similar mating, gallery constructing, and oviposition activities observed in *Ips avulsus*.)

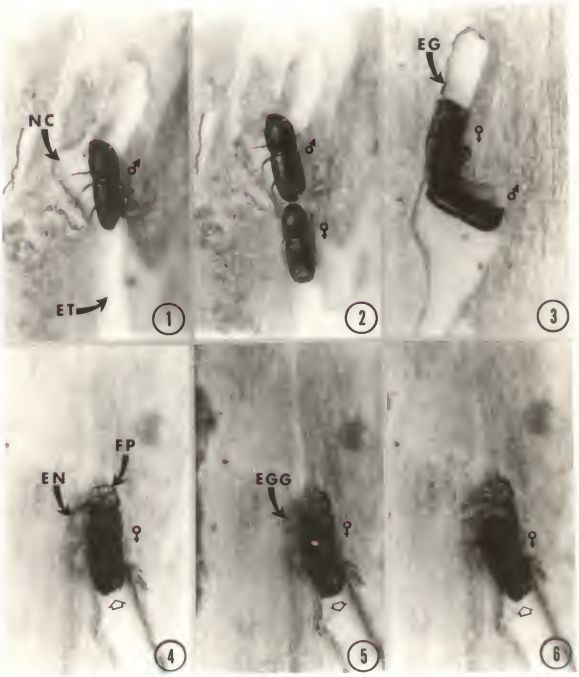

NC
♂
ET
1
♂
♀
2
EG
♀
♂
3
EN
FP
♀
4
EGG
♀
5
♀
6

PLATE IV

Figure A. Male *Ips avulsus* preceding female into nuptial chamber.

Figure B. Female *Ips avulsus* assisting male in construction of nuptial chamber.

Figure C. Male removing frass from nuptial chamber.

Figure D. Copulation in nuptial chamber

Figure E. Female constructing egg niche in egg gallery.

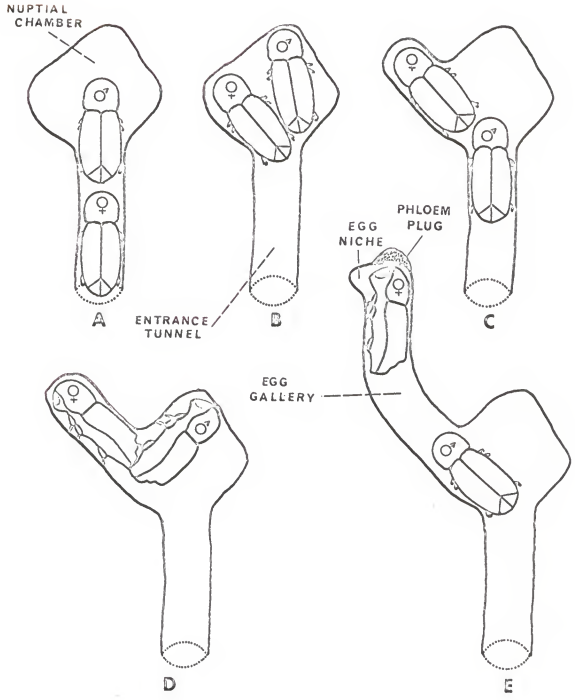

NUPTIAL CHAMBER
A
ENTRANCE TUNNEL
B
C
EGG NICHE
PHLOEM PLUG
EGG GALLERY
D
E

PLATE V

Figure F. Female backing out of egg gallery.

Figure G. Female turning around in nuptial chamber.

Figure H. Female backing into egg gallery.

Figure I. Female ovipositing in egg niche.

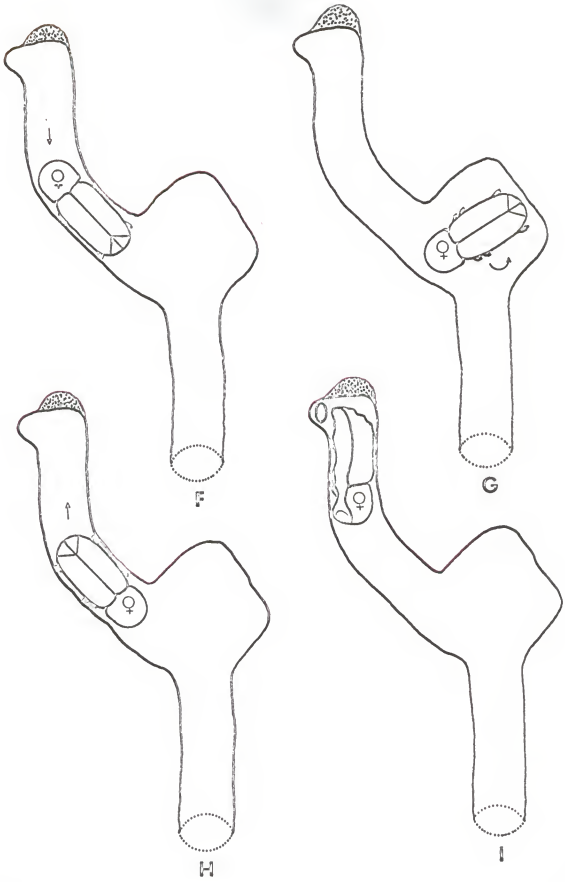
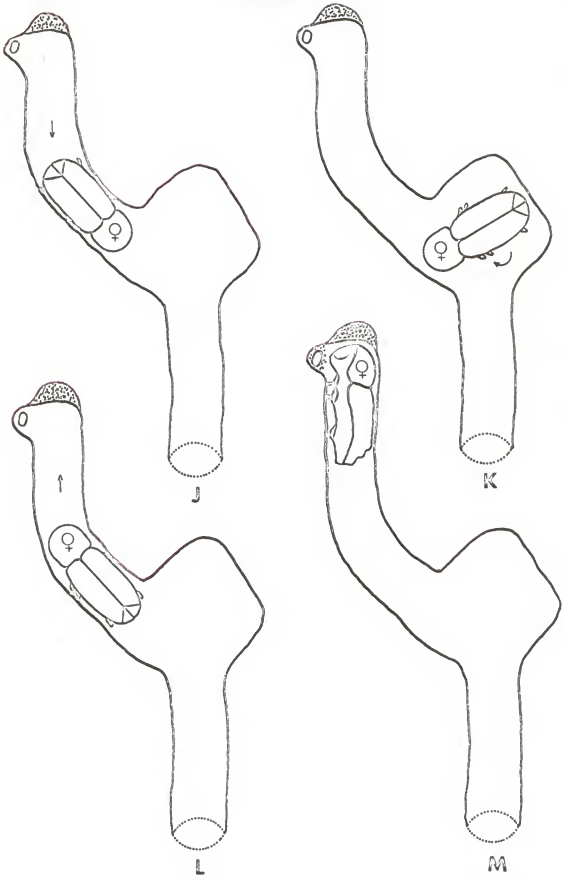

F
G
H
I

PLATE VI

Figures J, K, L. Female reversing her position and re-entering the egg gallery.

Figure M. Female packing phloem around the egg.


J
K
L
M

CHAPTER IV

FUNGI ASSOCIATED WITH *IPS AVULSUS* IN FLORIDA

Background

Although *Ips* bark beetles live in a nutrient-rich habitat, which supports growth of various kinds of micro-organisms, many of these beetles are found in constant association with specific fungi. For the purpose of discussing these latter ectosymbionts, three categories of fungi are designated. They are the blue-staining fungi, ambrosia fungi and yeasts.

Reviews of bark beetle-bluestain fungus associations have been given by Rennerfelt (1950), Mathiesen-Kaarik (1953), Francke-Grosmann (1963), and Mathre (1964). Most blue-staining fungi belong to one ascomycetes genus (*Ceratocystis*), or to the hyphomycetes genera (*Leptographium* or *Graphium*) which are known to be imperfect stages of *Ceratocystis*. Hunt (1956) revised the genus *Ceratocystis*, and all previous citations from the literature have been given in terms of his monograph. The genus *Ceratocystis* is noted for its association with both phloem-eating (bark) and wood-boring (ambrosia) beetles. The species of blue-staining fungi associated with *Ips* bark beetles are presented in Table I.

TABLE I

The Species of Fungi Associated with *Ips* Bark Beetles

Fungus	Beetles	References
	(Blue-stain fungi)	
Ceratocystis penicillata (Gros.) C. Moreau	*Ips typographus*	Grosmann (1931) Siemaszko (1939)
Ceratocystis ips (Rumb.) C. Moreau	*Ips sexdentatus* Boern.	Siemaszko (1939)
	Ips calligraphus (Germ)	Franche-Grosmann (1952)
	Ips avulsus (Eichh.)	Rumbold (1931)
	Ips grandicollis (Eichh.)	Rumbold (1931)
	Ips pini (Say)	Rumbold (1931)
	Ips lecontei Sw.	Ellis (1939)
	Ips oregoni Eichh.	Ellis (1939)
	Ips plastographus	Rumbold (1936)
	Ips ponderosae Sw.	Mathre (1964)
	Ips confusus Lec.	Mathre (1964)
	Ips emarginatus Lec.	Mathre (1964)
Ceratocystis brunneo-cilata (M-Daa) Hunt	*Ips sexdentatus*	Mathiesen (1951)

TABLE I—*Continued*

Fungus	Beetles	References
	(Blue-stain fungi)	
Ceratocystic clavata (Math.) Hunt	*Ips acuminatus* Gyll.	Mathiesen (1951)
	Ips sexdentatus	Francke-Grosmann (1952)
Ceratocystis polonia (Siem.) C. Moreau	*Ips typographus* L.	Siemaszko (1939)
Ceratocystis floccosa (Math.) Hunt	*Ips typographus*	Mathiesen (1950)
Ceratocystis albida (Math.) Hunt	*Ips typographus*	Mathiesen (1950)
Ceratocystis minuta (Siem.) Hunt	*Ips typographus*	Siemaszko (1939)
Ceratocystis montium (Rumb.) Hunt	*Ips emarginatus* Lec.	Rumbold (1941)
	Ips confusus	Mathre (1964)
	(Ambrosia fungi)	
Ambrosiella macrasporium (Fr.-Gr.) Batra	*Ips acuminatus*	Francke-Grosmann (1952)
Ambrosiella ips (Leach) Batra	*Ips sexdentatus*	Siemaszko (1939)
	Ips pini	Leach et al. (1934)

TABLE I—*Continued*

Fungus	Beetles	References
	(Ambrosia fungi)	
	Ips grandicollis	Leach et al. (1934)
	Ips oregoni	Batra (1967)
Dipodascus aggregatus Fr.-Gr.	*Ips acuminatus*	Batra (1967)
	(Yeast fungi)	
Hansenula beckii (Beck) Wickerham	*Ips typographus*	Beck (1922)
Pichia pini (Holst) Phaff	*Ips oregoni*	Holst (1936)
	Ips emarginatus	Holst (1936)
	Ips avulsus	Holst (1936)
	Ips grandicollis	Holst (1936)
	Ips calligraphus	Holst (1936)
	Ips confusus	Callaham and Shifrine (1960)
Hansenula capsulata Wickerham	*Ips confusus*	Callaham and Shifrine (1960)
	Ips oregoni	Callaham and Shifrine (1960)

TABLE I—*Continued*

Fungus	Beetles	References
	(Yeast fungi)	
Hansenula holstii (Shifrine & Phaff) Wickerham	*Ips oregoni*	Callaham and Shifrine (1960)
	Ips confusus	Callaham and Shifrine (1960)
Candida mycoderma (Reess) Lodder and vanRij	*Ips confusus*	Callaham and Shifrine (1960)
	Ips oregoni	Callaham and Shifrine (1960)
Candida curvata (Diddens & Lodder) Lodder and vanRij	*Ips confusus*	Callaham and Shifrine (1960)

The ambrosia fungi are those fungi which are regularly transported and implanted by insects into a suitable culture medium and develop into a growth which becomes the insects' essential food supply. The term ambrosia is that of Schmidberger (1836) who found salty crusts lining the tunnel walls of *Xyleborus dispar* F. on which the beetles were feeding. Taxonomically, most of the ambrosia fungi belong to the Endomycetales, Ophiostomatales and other ascomycetous orders and Fungi Imperfecti (Batra 1959). Recently, Batra (1967) has revised the primary ambrosia fungi associated with beetles to include four genera of Tuberculariaceae, *Ambrosiella* Brader, *Raffaelea* V. Arx and Hennebert, *Monacrosporium* and *Phialophoropsis* Batra and two genera of Endomycetales, *Ascoidea* Bref. and Lindau and *Endomycopsis* Dekker.

Species of ambrosia fungi found with *Ips* bark beetles are presented in Table I.

Information on yeasts associated with bark beetles has been reviewed by Shifrine and Phaff (1956) and Callaham and Shifrine (1960). Most work deals with a few yeasts commonly found with certain bark beetles. The most widely used classification of yeasts is that of J. Loddar, W. Ch. Slooff and N. J. W. Kregervan Rij, reported by Cook (1958).

They classify the genera reported herein as follows:

Family - Saccharomycetaceae

Subfamily - Saccharomycetoideae

Tribe - Saccharomyceteae - Genera *Pichia*, and *Hansenula*.

Family - Cryptococcaceae

Subfamily - Cryptococcoideae - Genus *Candida*

The species of yeasts associated with *Ips* bark beetles are presented in Table I.

The general conclusion of the workers is that when these three kinds of ectosymbiotic fungi are transmitted into a tree by beetles, they render the tree a more suitable medium for the beetles. It is not clear in what way bark beetles are benefited as only correlative evidence exists. Certain *Ceratocystis* species such as *C. ips* and *C. minor* are almost exclusively disseminated by bark beetles (Leach et al. 1934) (Mathiesen 1950). The action of the beetles on the inner bark apparently creates conditions favorable for fungal growth and *C. minor* failed to become established in trees when southern pine beetle, *Dendroctonus frontalis* Zimm., broods were unsuccessful in developing (St. George and Beal 1927). In pathogenicity trials, Mathre (1964) and Nelson (1934) were unable to obtain infection by *C. ips* or *C. minor* when the inoculum was introduced through small holes in the bark intended to simulate bark beetle attacks.

Bluestain fungi rapidly spread longitudinally and radially in infected trees, but tangential spread was slow and took place through tangentially extended larval galleries of bark beetles (Grosmann 1930, Leach et al. 1934, Nelson and Beal 1929).

Bluestain fungi established in a host impede water conduction (Mathre 1964, Nelson 1934) and contribute directly to the death of the tree. Many workers consider that the reduction of water content of trees infected by the fungi is necessary for successful beetle brood development (Bramble and Holst 1940, Caird 1935, Craighead 1928, Mathre 1964, Nelson 1934, Nelson and Beal 1929). Leach et al. (1934) observed that the *C. ips* perithecia were eaten by *Ips pini* and *Ips grandicollis* adults, but found that the ascospores were not digested in passing through the gut. Hodges et al. (1968) found that growth of *C. minor* in inner pine bark resulted in a marked increase in insoluble protein, a condition possibly favorable to development of the southern pine beetle. Barras and Hodges (1969) found *C. minor* growth greatly reduced the level of reducing-sugars in inner pine bark, but suggested that normally, when adult beetles and microorgansims were introduced at the same time, the sugar level was adequate for both. They suggested that a low C/N ratio in blue-stained

phloem might prevent brood development or adversely affect male or female reproductive processes. Barras (1970) further reported that *C. minor* acting alone was detrimental to southern pine beetle development, but that bluestain fungus growth and perithecia production were inhibited in the phloem when the beetle and other associated micro-organisms were present.

Hetrick (1949) observed a southern pine beetle infestation in which the trees showed no signs of blue-staining, yet the trees died rapidly and apparently normal broods developed. Holst (1937) suggested that bluestain fungi were not essential for at least partial single-generation development (from egg to adult) when he reared small numbers of the southern pine beetle, *I. calligraphus*, and *I. grandicollis* on altered (autoclaved) phloem strips. Grosmann (1930) noted that *I. typographus* larvae moved ahead of the spread of bluestain fungus in infested bolts and very little, if any, fungal material was consumed by the larvae. Franklin (1970) indicated that southern pine beetle larvae mined but did not grow in *C. minor*-stained phloem. He speculated that the fungus might have a toxic and repellent effect on the larvae and a repellent effect upon adults.

Person (1931) and Lu et al. (1957) found that yeast and yeast-infected phloem attracted bark beetles in some experiments. Rumbold (1941) studied the yeasts carried by bark beetles and found that they apparently promoted the growth of *Ceratocystis* fungi on wood, and Webb (1945) proposed that certain yeasts accelerated the growth of ambrosia fungi. Francke-Grosmann (1967) mentioned that the yeasts (as a source of vitamins and nutrients) may play a more prominent role in the ecology of bark beetles than the bluestaining fungi. Callaham and Shifrine (1960) suggested that the yeast floras of many species of bark beetles should be analyzed; that studies should be made of geographical and seasonal variations in yeast floras and that the role of yeast in the production of bark beetle attractants and in the nutrition of bark beetles should be clarified.

Due to the uncertainty of the exact nature of the interrelations between bark beetles and fungi, Yearian (1966) initiated a project to study the *Ips* bark beetles occurring in Florida and their bluestain fungus associates. His results showed reproduction in pine bolts was significantly greater in wild *Ips avulsus* bark beetles compared either with beetles reared aseptically on artificial medium and inoculated with bluestain fungus or aseptic beetles.

He suggested that some organism(s) other than *C. ips* promoted this increase in fecundity and brood development.

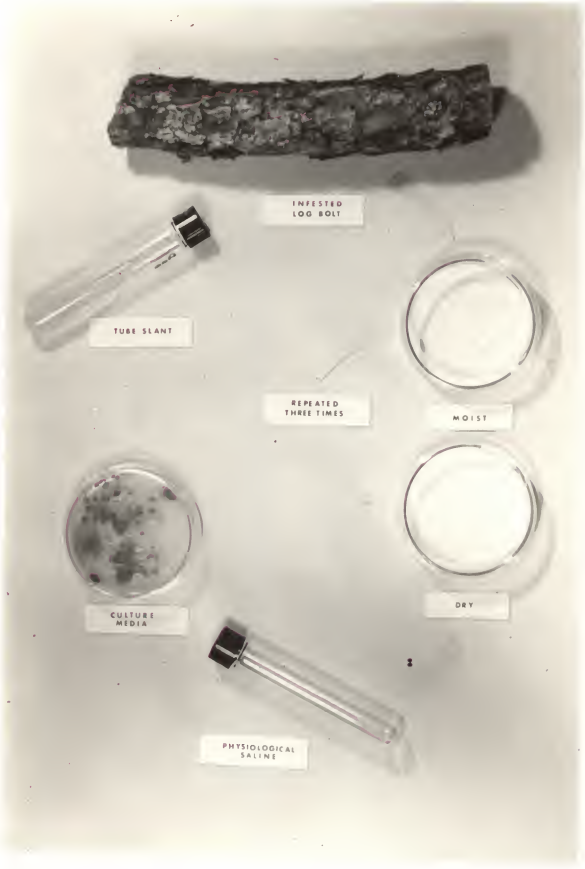
In this study emphasis was placed on the isolation and identification of fungi associated with *I. avulsus* infesting *P. elliottii* var. *elliottii*.

Methods

The fractional sterilization procedure developed by Francke-Grosmann (1956b) was used to isolate fungi from adult beetles. Beetles were collected as they emerged from *P. elliottii* in Austin Cary Forest on June 10, 1968, September 2, 1968, December 30, 1968, and March 15, 1969. One hundred beetles were collected on each date and placed in individual sterile petri dishes lined with moist filter paper for 12 hours and then transferred to dishes with dry filter paper for the same period. This procedure was repeated 3 times. The object of this procedure was to germinate the spores adhering to the insect's exoskeleton during the 12 hours moist periods and then kill the germinated spores through desiccation during the dry periods. Fifty beetles that were alive after the final exposure to dry conditions were crushed in physiological saline and streaked over the surface of various culture media (Plate VII).

PLATE VII

Figure 1. Illustration of a modified fractional sterilization procedure developed by Francke-Grosmann (1956). One hundred beetles were collected emerging from infested log bolts and placed in individual petri dishes lined with moist filter paper for 12 hours and then transferred to dishes with dry filter paper for the same period. This procedure was repeated three times. 50 beetles that were alive after the final exposure to dry conditions were crushed in physiological saline and streaked over the various culture media. All incubated media were incubated at 30°C for 24 hours. Subcultures were then made of the various fungi and pure cultures of each organism were inoculated on tube slants and held at 15°C prior to identification.


INFESTED
LOG BOLT
TUBE SLANT
REPEATED
THREE TIMES
MOIST
DRY
CULTURE
MEDIA
PHYSIOLOGICAL
SALINE

Surface sterilization of eggs, larvae and pupae was accomplished by washing the specimens for 5 minutes in the following modification of the sterilizing solution given by Vanderzant and Davich (1956); 0.25 grams mercuric chloride, 6.5 grams sodium chloride, 1.25 milliliters hydrochloric acid, 250 milliliters 95% ethyl alcohol and 750 milliliters distilled water (Yearian 1966). The wash was followed with a 3-minute rinse in sterile distilled water. Fungus isolations from the specimens were made by crushing them with sterile forceps in physiological saline and streaking the fragments on the surface of various media.

Phloem plugs were teased from the egg niches, crushed in physiological saline and streaked on the surface of malt agar, potato destrose agar and a pine phloem agar developed by the author (Appendix).

All inoculated media were incubated at 30° C for 24 hours. Subcultures were then made of the various fungi and pure cultures of each organism were inoculated on tube slants and held at 15°C.

Results and Discussion

Four ectosymbiotic fungi were found to be frequently associated with *I. avulsus* in Florida (Table II). They included *Ceratocystis ips* (Rumbold) C. Moreau, the

TABLE II

Relative Frequency (Percentage) of Fungi Isolated from *Ips avulsus**

Species of Fungi**	Month Isolated	Stage of Development						
		Egg	1	2	3	Pupa	Adult	
(BS) *C. ips*	Mar.	0	0	85	100	0	100	100
	June	0	0	76	100	0	100	100
	Sept.	0	0	74	100	0	100	100
	Dec.	0	0	60	76	0	74	78
(Y) *H. holstii*	Mar.	0	87	100	100	0	100	100
	June	0	80	100	100	0	100	100
	Sept.	0	74	100	100	0	100	100
	Dec.	0	92	100	100	0	100	100
(Y) *P. pini*	Mar.	0	79	100	100	0	100	100
	June	0	55	100	100	0	100	100
	Sept.	0	60	100	100	0	100	100
	Dec.	0	95	100	100	0	100	100
(A) *Ambrosiella* sp.	Mar.	0	0	0	0	0	25	30
	June	0	0	0	0	0	0	0
	Sept.	0	0	0	0	0	0	0
	Dec.	0	0	0	0	0	70	75

* Based on 50 specimens from each life stage.

** (BS) bluestain fungus, (Y) yeast fungus, (A) ambrosia fungus.

consistently associated bluestain fungus, two species of yeasts, *Hansenula holstii* Wickerham and *Pichia pini* (Holst) Phaff and an ambrosia fungus tentatively identified by the author as a new species of *Ambrosiella* Brader ex v. Arx & Hennebert.

The following are descriptions of the fungi isolated in this study.

a. *Ceratocystis ips* (Rumbold) C. Moreau

Ceratocystis ips was first described from the eastern United States in association with *Ips avulsus* by Rumbold (1931). Davidson (1935) and Verrall (1939) found *C. ips* to be an important cause of log and lumber stain on pine in the South. It is associated with beetle attacks in the logs and continues to develop in lumber cut from infested logs. It may be spread to uncontaminated lumber (especially rough lumber with bark intact) by insects or by contact with contaminated mill equipment or lumber (Verrall 1941).

The following characteristics are based largely on descriptions by Rumbold (1931) and Hunt (1956). No variations from their descriptions were found in the fungus isolated in this study. The perithecia are superficial, the bases black, appearing brown to black under the microscope, globose, 120-22μ in diameter, ornamented with pale

brown, thin-walled, septate hyphae attached to all sides, some bearing conidia, others continuous with the vegetative mycelium, 2.5-5μ in diameter; necks black, sometimes bent, up to 1100μ long, 27-40μ in diameter at the base and 10-25μ at the tip (Plate VIII, Figures 1, 3); ostiolar hyphae lacking (Plate VIII, Figure 4); asci not seen; ascospores appearing rectangular due to flanges formed by the gelatinous sheath, 3-5 × 1.5 - 2.5μ (Plate VIII, Figure 2).

Cultures at first produce a loosely interwoven, sparse, hyaline mycelium, turning brown in about 5 days and finally black in 2 weeks; sometimes areas remaining brown due to sectoring; some isolates with mycelium completely appressed, others with a matted aerial mycelium; conidia often absent, when present, appearing in 3-5 days; perithecia forming after 1-2 weeks, maturing in about 1 week after appearance; growth rapid, 35 mm.; odor not distinctive. Aerial hyphae hyaline at first, turning pale brown to almost black with age, occurring singly and in fascicles of 2-10 hyphae, branched and interwoven, thin-walled, regularly septate; some cells swollen, 1-10μ in diameter; submerged hyphae similar; hyphae in wood thin-walled, pale brown to black, sometimes absent, varying from single, septate, hyaline to pale brown hyphae with conidia borne at the tips of 2-5 ramified branches to *Graphium*-like

structures with a definite stalk and head of ramified branches, the latter up to 300μ high, up to 20μ in diameter at the base and flaring to a head up to 250μ in diameter (Plate VIII, Figures 6,7); conidia hyaline, cylindric to ovoid, sometimes truncate, 4-8 × 1.5-3μ (Plate VIII, Figure 5).

Based on the *Leptographium*-like imperfect stage, Goidanich (1936) placed *C. ips* in his new genus *Grosmannia*. Siemaszko (1939) did not recognize *Grosmannia* and further pointed out that *C. ips* produces *Graphium*-like conidiophores. The coremia of *C. ips* represent a transitional form between *Leptographium* and *Graphium* and in the strict sense the imperfect stage cannot be placed in either of the above genera (Hunt 1956).

b. *Hansenula holstii* Wickerham

Hansenula holstii has never been found in nature in the diploid state but the haploid form is isolated frequently in North America, Europe and Asia from the frass of many scolytid beetles infesting conifers and from the gums of *Prunus* species (Farmer 1965). *Hansenula holstii* was first described by Shifrine and Phaff (1956) from *Ips emarginatus* infesting *Pinus jeffreyi* Grev. and Balf. This is believed to be the first report of *H. holstii* isolated from *Ips avulsus* and *Pinus elliottii* var. *elliottii*.

PLATE VIII

Figure 1. Perithecia of *Ceratocystis ips* growing on phloem plug. Note round black perithecial bases and elongate necks. Photographed ×2.5 and enlarged. (photo by author)

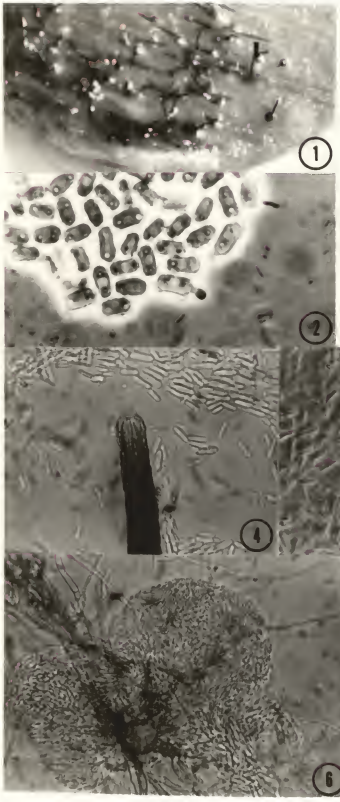
Figure 2. Mature ascospores. Rectangular flanges formed by the gelatinous sheath not shown in this photograph. Photographed ×970 and enlarged. (photo by author)

Figure 3. Mature perithecium. B, base; N, neck. Photographed ×100 and enlarged. (photo by author)

Figure 4. Neck of perithecium. Note absence of ostiolar hyphae. Photographed ×150 and enlarged. (photo by author)

Figure 5. Cylindrical conidia. Photographed ×440 and enlarged. (photo by author)

Figure 6-7. Conidiophores. This is the *Leptographium*-like imperfect stage. Photographed ×440 and enlarged. (photo by author)


1
2
N
B
3
4
5
6
7

The following characteristics of *H. holstii* in Florida corresponded to those given by Farmer (1965). Hyphae and pseudohyphae up to 2.5 mm. long; asporogenous and haploid in nature except very rarely heterothallic; blastospores ellipsoidal to short cylindrical, sometimes spherical and ovoidal; blastospores may end in long pointed tapers from one to several times the length of the cell; blastospores on hyphae vary from 1.7 × 2.6 to 3.4 × 6.0μ, those from edge of nonfilamented colonies may vary from 0.9 × 1.7 to 3.4 × 7.2μ; ascospores of conjugated haploids, hat-shaped 2.0 to 2.5μ, usually two per ascus.

Colonies are usually mucoid, more watery than viscous, often with a fringe of hyphae which develops with aging.

This yeast assimilates glucose, galactose, L-sorbose, maltose, sucrose, cellobiose, trehalose, melezitose, soluble starch, xylose, L-arabinose, D-arabinose, D-ribose, rhamnose. D-glucosamine hydrochloride, ethanol, glycerol, mannitol, sorbitol, alphamethylglucoside, salicin, pyruvate, succinate, citrate and nitrate. Glucose produces a gaseous fermentation; galactose is fermented weakly. Esters are not produced and an exogenous vitamin supply is required for growth in culture (Farmer 1965).

It produces extracellular phosphorylated mannans as capsular material which adhers to bark beetle exoskeletons, as previously discussed.

c. *Pichia pini* (Holst) Phaff

This organism was first described by Holst (1936). It is widely associated with both *Dendroctonus* and *Ips* species in *Pinus ponderosa* Laws., *P. monticola* Dougl., *P. contorta* Dougl., *P. lambertiana* Dougl., *P. echinata* Mill., *P. strobus* L., and *P. virginiana* Mill., in the United States (Farmer 1965) and has been reported by Robinson (1962) from *P. contorta* Dougl. in British Columbia, Canada. Rumbold (1931) isolated this yeast from *Ips avulsus* attacking *P. echinata*. However, this appears to be the first report of *Pichia pini* infecting *Pinus elliottii* var. *elliottii*.

The following characteristics are given by Farmer (1965). True hyphae are not developed in culture; haploid or diploid; homothallic, sporogenous, producing up to 4 ascospores; free spores irregularly arranged in clusters in culture; cells in malt extract culture round to ellipsoidal or egg-shaped, 2.0μ to 6.4μ × 2.5μ to 7.3μ; ascospores 1.6μ to 2.0μ × 2.3μ to 3.0μ; reproduces asexually by budding of cells or spores; spores hat-shaped, germinate without fusion, commonly by budding, sometimes by germ tube.

The colonies are white at first frequently becoming fawn-colored or pinkish with white radial sectors persisting; mucoid growth moderate with spreading surface smooth, becoming papillate with irregular lobes at edge.

This yeast does not assimilate nitrates; ferments glucose, fructose and mannose with production of acid and gas; assimilates glucose, trehalose, L-arabinose, xylose, cellobiose, D-ribose, rhamnose, ethyl alcohol, glycerol, erythritol, mannitol, sorbitol, salacin, calcium 2-Kelogluconate, pyruvate and citrate. None of the species assimilate nitrate, but they produce phosphomannan extracellularly as do the *Hansenula* species (Wickerham and Burton, 1961).

d. *Ambrosiella* sp.

One of the four fungi found to be closely associated with *Ips avulsus* in Florida is apparently an undescribed ambrosia fungus which closely resembles *Ambrosiella ips* (Leach, Orr and Christensen) Batra. However, the fungus in Florida does not cause a bluestain in wood, and the spores are cylindrical rather than pyriform. When this fungus was present (winter and spring, Table I), it was most often observed fruiting in the pupal chambers and larval tunnels. Only the conidial stage was found which consisted of white sporodochia (Plate IX, Figures 2-6). Sporulation appeared to begin shortly after pupation and

the newly formed beetle pupae often appeared to be covered with the white conidia. These cylindrical conidia (Plate IX, Figure 1) were present on the ends of conidiophores (Plate IX, Figure 3) and formed a compact cushion (Plate IX, Figures 4-5).

This fungus appears to belong to *Ambrosiella* Brader ex v. Arx and Hennebert (1965), a genus which is characterized by monilioid conidiophores ending with a terminal conidium and loosely arranged in sporodochia without a conspicuous stroma (Brader, 1964). Until a more detailed study of this fungus can be made, it will be tentatively classified as *Ambrosiella* sp.

The following is a brief technical description of this fungus. Conidia cylindrical, ranging from 2.0μ to 2.5μ in width and 5.5μ to 4.5μ in length, borne singly and successively at the ends of unbranched septate conidiophores; sporodochia white and waxy but not mucilaginous. Young colonies on malt agar colorless later changing to light brown; young hyphae hyaline, old hyphae light brown; mycelium 1.5μ to 4.5μ in diameter; sporodochia common in cultures. This fungus was isolated from the phloem of *Pinus elliottii* var *elliottii* infested with *Ips avulsus* at Austin Cary Forest, Gainesville, Florida.

e. Miscellaneous fungi

The following fungi were periodically isolated from *I. avulsus* in Florida. Based on their random

PLATE IX

Figure 1. Cylindrical conidia of *Ambrosiella* sp. Photographed ×440 and enlarged. (photo by author)

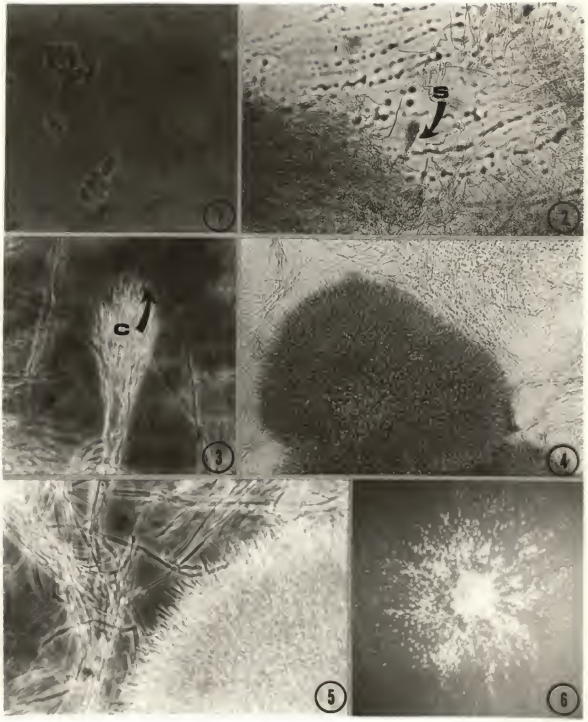
Figure 2. Developing sporodochium. S, sporodochium. Photographed ×100 and enlarged. (photo by author)

Figure 3. Developing sporodochium. Note conidia attached to conidiophores. C, conidia. Photographed ×250 and enlarged. (photo by author)

Figure 4. Mature sporodochium. Photographed ×100 and enlarged. (photo by author)

Figure 5. Portion of mature sporodochium. Photographed ×250 and enlarged. (photo by author)

Figure 6. Colony on malt agar. (×2) (photo by author)


1
S
2
C
3
4
5
6

occurrence in culture, they were not considered to be ectosymbionts in this study.

Penicillium spp.
Peniophora gigantea (Fr.) Massee
Trametes serialis Fries
Trichoderma lignorum Pers.
Hansenula sp.
Saccharomyces sp.

f. Bacteria

Most workers have recorded several species of bacteria usually present in the phloem-bark beetle-fungus complex. D. P. Jouvenaz (personal communication) tentatively identified two species of bacteria associated with *Ips avulsus.* The first, *Serratia marcescens* Bizio, only occasionally found in culture, was considered to be pathogenic to *Ips calligraphus* (Jouvenaz and Wilkinson, 1970). The other bacterium tentatively identified as *Pseudomonas* sp. was universally present and should be considered in any further study of the bark beetle-microorganism complex.

Seasonal Variation

Isolations from fifty of each of the life stages of *I. avulsus* in March, June, September and December showed marked seasonal variation in their fungal floras (Table II). Surface-sterilized eggs and pupae were

internally free of any fungi isolated in this study. These data correspond to that of Yearian (1966). First stage larvae were free of *C. ips* and *Ambrosiella* sp., but both *H. holstii* and *P. pini* were isolated from first stage larvae on all dates. The highest incidence, 92 and 95% respectively, occurred in December. Yeasts were isolated from all second and third stage larvae on all dates.

The relative frequency of *C. ips* isolated from adult beetles was 100% in March, June and September. However, only 74% of the males and 78% of the females were contaminated in December. Yearian (1966) isolated *C. ips* from 85% of the males and 83% of the females. The slight difference in results could be attributed in part to seasonal variation. Yearian did not indicate the dates on which he obtained his specimens.

Both yeasts were isolated from adult beetles on all dates (100%). The yeast flora of *I. avulsus* consisting of *P. pini* and *H. holstii*, which were universally associated with the beetles, and several unidentified yeast species which were randomly isolated from this beetle correspond to reports by Grosmann (1930), Lu et al. (1957), Shifrine and Phaff (1956) and Callaham and Shifrine (1960) for other bark beetles. However, only one type of yeast was usually found on each plate, occasionally two yeasts were isolated, and rarely three were present. The universal association

of yeasts, both internally and externally, corresponds to reports by Leach et al. (1934), Caird (1935) and Farmer (1965). Any seasonal variation in the composition of the yeast flora has not been reported in the literature.

Ambrosiella sp. was isolated from male and female beetles, 25 and 30% respectively, in March, and 70 and 75% in December. These results may correspond to those of Batra (1963). He noted that a greater proportion of ambrosia cells isolated from spring and summer broods of ambrosia beetles did not germinate. He hypothesized the lack of germination of inoculum from late spring to fall as due to several factors: (1) It is possible that a dormancy period is necessary for the ambrosia cells prior to germination, (2) lack of proper nutrients in media used for isolation, or, more probably, (3) the absence of certain nutritional stimuli (especially nitrogen) from mycangia could be an important factor.

Further studies are needed to clarify the possible role of nitrogen in the regulation of the fungi. Hodges et al. (1968) points out that care must be taken when interpreting tests in which an organism is grown on an ill-defined medium such as the inner bark of pine. Chemical changes in the medium over a period of time may not be due entirely to the activity of the organism. Also, certain beetles

show preferences for certain parts of a tree (Thatcher 1960), and those preferences may be related to variation in host tissue composition.

CHAPTER V

INTERACTIONS OF FUNGI ASSOCIATED WITH *IPS AVULSUS*

Background

Much has been written concerning the effects of certain fungi on establishment of bark beetles in standing trees, but little has been published on the interactions of fungi and their phloem and/or xylem substrate. Barras (1970) found that the growth and perithecia production of *C. minor* were inhibited in the phloem when it was present with *Dendroctonus frontalis* and other associated micro-organisms. The inhibition mechanism was unknown, but oleoresins (Cobb et al. 1968) or mycangial fungi (Barras and Hodges 1969, Hodges et al. 1968) may have been directly involved. Also, trials by Barras (1969) have shown an antagonistic reaction between *Penicillium implicatum* Biourge and both *C. minor* and *C. ips*. Rumbold (1941) studied the fungi carried by certain bark beetles and noted that several species of yeasts were found in association with these beetles. These yeasts appeared to promote the growth of *Ceratocystis* spp. on wood. Webb (1945) and Baker (1963) suggested that the yeasts present with certain ambrosia beetles promoted the growth of the principal ambrosia fungus.

To understand the microbial interaction in any environment requires four basic studies (Vaartaja and Salisbury 1965); (1) exploration of the potential effects of the commonly present organisms on each other, at first in pairs; (2) then exploration in other combinations; (3) a search for what determines the role of these potential effects in experiments; and (4) studies in the natural environment.

The work described herein is a contribution toward (1), (2) and (3) concerning the *Ips avulsus*—fungi association.

Methods

To determine the effects between the fungi isolated in this study, they were first paired in various combinations by inoculating two isolates on malt agar at opposite edges of a standard (9 cm.) petri dish. Each dish was inoculated from malt agar cultures divided into circular pieces with a sterile No. 4 cork borer. One petri dish represented a treatment. Treatments were replicated 10 times and paired cultures were incubated for 20 days under fluorescent light at 30°C and 60-70% humidity.

The clearly identifiable effects were based on comparison of growth of paired isolates of the same organism and were classified as follows: (0) no effect;

(S) sporulation affected, (-) decrease, (+) increase (based on maturation of fruiting bodies) (±25%); (D) decrease in growth rate after contact (more than 1 cm. per day); (ST) stimulation of growth rate after contact (more than 1 cm. per day); (A) antagonistic effect on growth from 1-10 mm. The effects were recorded daily for 20 days.

To determine if any of the test fungi produced diffusable substances which acted as growth regulators, sterilized U-shaped drying tubes (Fisher Scientific Company, Catalog No. 9-240) were filled to a depth of 5 cm. with 3% malt agar. The agar was allowed to solidify and then was inoculated with two organisms placed on opposite sides of the tube. These tubes were also inoculated with circular pieces from malt agar cultures with a sterile No. 4 cork borer. One U-tube represented one treatment. Each treatment was replicated 10 times and incubated as in the paired fungus tests; the clearly identifiable effects were also recorded as in the paired fungus test.

Interactions between the various fungi on the inner bark of *Pinus elliottii* var *elliottii* were determined by use of a "phloem plug" technique in which an aseptic phloem plug was placed in agar and innoculated with two fungal isolates. This technique was as follows: (1) a 3' fresh log bolt was brought into the laboratory; (2) all loose bark scales were removed with a draw knife and the bolt was

washed down with 70% ethyl alcohol; (3) the outer bark was removed to expose the phloem; (4) the phloem was washed with 70% alcohol; (5) plugs of phloem were removed with a sterile No. 6 cork borer and placed in a sterile petri dish; (6) 5% water agar was poured into sterile petri dishes to a depth of 1 cm. and allowed to cool to 50°C; (7) one phloem plug was rinsed in 70% ethyl alcohol, flamed, and placed in the center of each petri dish; (8) the agar was allowed to solidify. Each dish was then inoculated with one pair of fungi. One dish represented one treatment. Only one log bolt was used as a source of phloem for each replication to reduce variability of the substrate. Each treatment was replicated 10 times. Observable growth was rated on a scale of 1 to 5. Greater than 40% reduction in growth compared to paired isolates of same organism was rated 1, 21-30% = 2, 11-20% = 3, 6-10% = 4 and 0% -5% = 5.

The effects of the paired fungi on *C. ips* perithecial production were determined by counting all mature exuding perithecia and calculating the mean number of perithecia per 10 replications. One phloem plug represented one treatment. It was recognized that there probably were other perithecia present which were mature from the aspect of having mature ascospores in them but which had not begun to exude the ascospores at the time of counting. The presence of exuded ascospores was the sole criterion of maturity.

Results and Discussion

Interactions of Paired Fungal Cultures on Malt Agar Plates and U-tubes

Decreased growth rates and/or sporulation were the most common effects of *Ambrosiella* sp. on *C. ips* on malt agar plates and U-tubes under the conditions of these tests, while there was no apparent effect on yeast growth rate or sporulation (Table III). There was no evidence that diffusable growth regulators were produced by any of the test fungi. This suggests that inhibition of growth and sporulation might have resulted from competition for nutrients and/or a weak antibiosis not detectable by these tests.

Effects of Test Fungi on C. ips Growing on Phloem Plugs in Agar

Results of the test to determine the effect of test fungi on *C. ips* perithecial production using the phloem plug technique are presented in Table IV. This test showed that *Ambrosiella* sp. significantly ($P = 0.01$) reduced *C. ips* perithecial production on all dates compared to *C. ips* with yeasts and *C. ips* alone. Also, the yeasts, *H. holstii* and *P. pini* significantly reduced ($P = 0.1$) perithecial production on day 12.

The interactions of test fungi in reference to growth rate on phloem plugs are presented in Table V. Again,

TABLE III

Interaction of Paired Fungal Cultures on Malt Agar Plates and U-Tubes*

Effect By***	Effect** On			
	Ambrosiella sp.	*C. ips*	*P. pini*	*H. holstii*
(A) *Ambrosiella* sp.	-----	(D) (S) (-)	(0)	(0)
(BS) *C. ips*	(D)	-----	(0)	(0)
(Y) *P. pini*	(D) (S) (-)	(D) (S) (-)	---	(0)
(Y) *H. holstii*	(D) (S) (-)	(D) (S) (-)	(0)	---

* Data on paired fungal cultures on malt agar plates and U-tubes were identical, and for the sake of brevity, are presented in one table.

** (D) decrease in growth rate after contact (more than 1 cm. per day), (S) sporulation affected, (-) decrease, (+) increase, (0) no effect.

*** (A) Ambrosia fungus, (BS) Blue Stain fungus, (Y) Yeast.

TABLE IV

Effects of Test Fungi on Perithecial Production of *Ceratocystis ips* Growing on Phloem Plugs in Agar

Effect By**	Effect on *C. ips* Perithecial Production		
	6 Days	12 Days	18 Days
	(Mean Number of Mature Perithecia)*		
(A) *Ambrosiella* sp.	6.6a	8.8a	18.6a
(Y) *H. holstii*	48.3b	72.6b	147.0b
(Y) *P. pini*	41.8b	69.2b	139.4b
(BS) *C. ips*	46.6b	97.5c	166.3b

* Mean number of mature perithecia per 10 replications. Means followed by same letters are not considered significantly different at the 1% level according to Duncan's multiple range test.

** (A) Ambrosia fungus, (BS) Bluestain fungus, (Y) Yeast.

a decrease in growth rate was the most common effect taking place. Both yeasts reduced the growth of *Ambrosiella* sp. In turn, *Ambrosiella* sp. reduced *C. ips* vegetative growth. None of the fungi in any combination were stimulated to increase their growth rate in this study. These results do not agree with those of Rumbold (1941), Webb (1945) and Baker (1963). Rumbold reported that certain yeasts appeared to promote bluestain fungus perithecial production, while

TABLE V

Growth Rates of Paired Fungi on Phloem Plugs in Agar

Effect By*	Effect on			
	Ambrosiella sp.	*C. ips*	*P. pini*	*H. holstii*
	(Growth Rating)**			
(A) *Ambrosiella* sp.	5	2	4	4
(BS) *C. ips*	2	5	4	4
(Y) *P. pini*	2	4	5	5
(Y) *H. holstii*	2	4	5	5

* Observable growth was rated on a scale of 1 to 5. Greater than 40% reduction in growth compared to paired isolates of same organism was rated 1, 21-30% = 2, 11-20% = 3, 6-10% = 4, 0%-5 = 5.

** (A) Ambrosia fungus, (BS) Blue Stain fungus, (Y) Yeast.

Webb and Baker noted that yeasts stimulated the growth of ambrosia fungi. No data were presented to support their observations, however.

It is hypothesized that an inter-dependency for nutrients, especially organic nitrogen, was responsible for the reduced growth rates and sporulation noted in this study. Campbell (1958) found that *C. ips* produced more perithecia on an asparagine-nitrate medium than on asparagine or calcium nitrate medium alone. He hypothesized that the fungus can utilize nitrate nitrogen when an organic nitrogen source furnishing an amino or amide group is available for the formulation of essential amino acids.

Abrahamson (1969) found that certain ambrosia fungi grew better on organic nitrogen sources than on an inorganic nitrogen source. In this study, when *Ambrosiella* sp. was accidentally exposed by the author to gaseous amonia produced by bacterial culture, its vegetative growth rate was greatly increased. Farmer (1965) noted that in addition to sufficient carbohydrates, yeasts require nitrogenous material for their metabolism. He found a significant increase in the relative amounts of nitrogen in non-infected phloem as compared to yeast-infected phloem.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Development of southern forests toward a pine monoculture could confront foresters and entomologists with the most serious forest insect control problem in the United States: that posed by bark beetles in southern pines.

The emphasis on integrated control measures to control pest populations points out the need to examine the life cycles of these insects including bark beetle-fungi associations. The present study of *Ips avulsus* and its associated fungi presents several contributions to this need.

Ips avulsus is closely associated with various fungi, and these fungi are introduced into trees by this beetle. In this study various mechanisms which aid in dissemination of these fungi by *Ips avulsus* are described.

Aspects of the life cycle of *Ips avulsus* are discussed with special elaboration on reproductive and feeding behavior and relative possibilities for fungus transmission.

The ectosymbiotic fungi associated with *Ips avulsus* in this study include: *Ceratocystis ips*, a common bluestain

fungus, two yeasts, *Hansenula holstii* and *Pichia pini* (*H. holstii* reported herein for the first time), and an ambrosia fungus, tentatively identified as an unknown species of *Ambrosiella* (also reported herein for the first time).

Other fungi periodically isolated from *I. avulsus* included: *Penicillium* spp., *Peniophora gigantica*, *Trametes serialis*, *Trichoderma lignorum*, *Hansenula* sp. and *Saccharomyces* sp. Based on their random occurrence in culture, they were not considered to be ectosymbionts of *I. avulsus*.

In addition, at least two species of bacteria, *Serratia marcescens*, considered to be pathogenic, and *Pseudomonas* sp., universally present in this association, are reported herein.

Little has been published on the interactions of fungi and their phloem substrate. Interactions of paired fungi isolated in this study showed that none of the fungi in any combination were stimulated to increase their growth rate. It was hypothesized that competition for nutrients, especially organic nitrogen, was responsible for the reduced growth rates and sporulation noted herein.

Some new and modified techniques are described as an aid to further studies of the bark beetle-fungi complex. These include methods for observing bark beetle behavior, and methods to evaluate fungus interactions.

The following conclusions are drawn as a result of these studies:

At least 3 fungus species, which included *Ceratocystis ips*, *Hansenula holstii*, and *Pichia pini*, were consistently associated with *Ips avulsus* in Florida. This paper apparently reports the first occurrence of *H. holstii* in association with *I. avulsus* in *Pinus elliottii* var *elliottii*. This is also the first report of *P. pini* associated with *P. elliottii* var. *elliottii*.

Another fungus, tentatively identified as an undescribed species of *Ambrosiella*, was frequently associated with *I. avulsus* during the winter and spring.

At least one species of bacterium was consistently associated with *Ips avulsus* in Florida.

No histological evidence was found of the existence of specialized structures for carrying fungi in this insect.

All of the fungi consistently associated with *Ips avulsus* possessed spores and cells which were imbedded in a gelatinous sheath. The investment of spores of the associated fungi by capsular sheaths suggests that the fungi are adapted for beetle transmission.

Based on this data and results presented by Yearian (1966), previously discussed, it is suggested that one or both of the yeasts and/or the bacteria

consistently isolated in this study are factors contributing to more rapid development and larger wild *I. avulsus* broods as compared to aseptic broods reared in the absence or presence of *Ceratocystis ips*. Although *Ambrosiella* sp. was not consistently isolated in this study it may be a factor in maintaining overwintering beetle populations.

Additional studies should include: (1) Identification of the bacterial associates and determination of their role in this association, (2) description of *Ambrosiella* sp. and investigation of the interactions contributing to the seasonal occurrence of this fungus in the complex, (3) a study of the possible significance of nitrogen in regard to the associations and (4) determination of the factor or factors which influence fecundity and brood development in this beetle. The latter would include rearing beetles in the presence and absence of the fungi and bacterial associates. If it is determined that one or more of the organisms isolated in this study are responsible for increased brood development and fecundity the biochemical significance of this association should be investigated.

APPENDIX

Carnoy's Fluid

100% ethyl alcohol	30 cc.
Glacial acetic acid	5 cc.
Chloroform	15 cc.

Haupt's Adhesive

Gelatin	1 g.
Phenol (Crystal)	2 g.
Glycerin	15 cc.
Distilled Water	100 cc.

Gram-Weigert Stain (Modified)

Solution I

ALUM HAEMATOXYLIN

Haematoxylin	1 g.
Ammonia alum (sat. aq. sol.)	100 cc.
Water	300 cc.
Potassium permanganate (0.25% aq. sol.)	10 cc.

A small thymol crystal

Pulverize haematoxylin, and dissolve it with the ammonia alum in the water with aid of heat. Cool and add potassium permanganate and then the thymol crystal. The stain is ready for use at once. As the stain ripens, it may be necessary to add a little of the saturated alum solution. Filter each day before using.

Solution II

EOSIN

Eosin ..... 5% aqueous solution

Filter each day before using.

Solution III

ANILINE METHYL VIOLET

Solution A

Absolute alcohol ..... 33 cc.
Aniline oil ..... 9 cc.
Methyl violet ..... In excess

Solution B

Saturated aqueous solution of methyl violet.

Mix 1 part of Solution A and 9 parts of Solution B not longer than 2 weeks before using. Filter each day before using.

Solution IV

LUGOL'S SOLUTION

Iodine	1 g.
Potassium iodide	2 g.
Distilled water	100 cc.

Stain sections for 3 minutes in alum haematoxylin solution. Rinse in running water. Stain 3 minutes in 5% eosin solution. Rinse off surplus stain in running water. Stain in Solution III (aniline methyl violet) for 5 minutes. Wash 1 minute in running water. Immerse in Lugol's solution for 1 minute. Rinse in running water. Drain off excess water, dry back of slide, and clear in a solution of 1 part xylol and 1 part aniline oil. Agitate slides until excess methyl violet stain is removed. Wash thoroughly in xylol to remove every trace of aniline oil, and mount in balsam.

Culture Media Used In This Study

MALT AGAR

Malt Extract, Difco	30 gr.
Bacto-Agar	15 gr.
Distilled Water	1,000 ml.

POTATO DEXTROSE AGAR

Bacto-Potato Dextrose Agar	39 gr.
Distilled Water	1,000 ml.

PHLOEM PINE AGAR

Phloem (*Pinus elliotti* var. *elliottii*)	1,000 gr.
Bacto-Agar	20 gr.
Distilled Water	1,000 ml.

To prepare this medium, 1,000 gr. of fresh phloem from *P. elliottii* var. *elliottii* were added to 1,000 ml. of distilled water and mascerated in a blender. The solid portion was removed by filtration through cheesecloth and discarded. Twenty grams of Bacto-Agar were then added to the liquid portion and autoclaved.

Fixation Schedule for Electron Microscopy
(Dr. H. C. Aldrich, Botany Dept., Univ. of Florida)

EITHER

1 hr. 2% $KMnO_4$ (unbuffered)

OR

1 hr. 2.5% Glutaraldehyde followed by 30 min. buffer wash.

1 hr. 1% buffered OsO_4. Use Sorenson's phosphate buffer.

THEN

Two 15 min. washes—one in buffer, one in H_2O.

THEN DEHYDRATE.

25% EtOH	5 to 15 min.
50% EtOH	5 to 15 min.
75% EtOH	5 to 15 min.
95% EtOH	5 to 15 min.
100% EtOH	5 to 15 min.
100% EtOH	5 to 15 min.
100% Acetone	15 min.
100% Acetone	30 min.
* 30% plastic - 70% Acetone	1 hr.
70% plastic - 30% Acetone	1 hr.
100% plastic (in capsule)	1 hr.

Put in 60° oven for 48 hours.

---

*Mollenhauer, M. H. 1964. Stain technique 39 (2): 111-114.

LITERATURE CITED

Abrahamson, L. P. 1969. Physiological interrelationships between ambrosia beetles and their symbiotic fungi. Ph.D. Thesis, University of Wisconsin, Madison.

Abrahamson, L. P. and D. M. Norris. 1966. Symbiotic interrelations between microbes and ambrosia beetles. I. The organs of microbial transport and perpetuation of *Xyloterinus politus*. Ann. Entomol. Soc. Amer. 59: 877-880.

Anderson, R. F. 1948. Host selection of the pine engraver. J. Econ. Entomol. 41: 596-602.

Arx, J. von and G. L. Hennebert. 1965. Deux champignons ambrosia. Mycol. Mycopath Appl. 25: 309-315.

Aslam, N. A. 1961. An assessment of some internal characteristics in the higher classification of the Curculionidae s. l. (Coleoptera). Trans. Roy. Entomol. Soc. Lond. 113: 417-489.

Baker, J. M. 1963. Ambrosia beetles and their fungi, with particular reference to *Platypus cylindrus* Fab. Symp. Soc. Gen. Microbiol. 13: 232-265.

Barras, S. J. 1969. *Penicillium implicatum* antagonistic to *Ceratocystis minor* and *C. ips*. Phytopathology 59: 1187-1190.

Barras, S. J. 1970. Antagonism between *Dendroctonus frontalis* and the fungus *Ceratocystis minor*. Ann. Entomol. Soc. Amer. 63: 1187-1190.

Barras, S. J. and J. D. Hodges. 1969. Carbohydrates of inner bark of *Pinus taeda* as affected by *Dendroctonus frontalis* and associated microorganisms. Can. Entomol. 101: 489-493.

Batra, L. R. 1959. A comparative morphological and physiological study of the species of *Dipodascus*. Mycologia 51: 329-355.

Batra, L. R. 1963. Ecology of ambrosia fungi and their dissemination by beetles. Trans. Kans. Acad. Sci. 66: 213-236.

Batra, L. R. 1967. Ambrosia fungi: a taxonomic revision, and nutritional studies of some species. Mycologia 59: 976-1017.

Beck, O. 1922. Eine neue endomyces-art, *Endomyces bisporus*. Ann. Mycol. 20: 219-227.

Blackman, M. W. 1915. Observations on the life-history of *Pityogenes hopkinsi*. N. Y. State Coll. Forest. 16: 11-66.

Brader, L. 1964. Etude de la relation entre le scolyte des rameaux du cafeier, *Xyleborus compactus* Eichh. (*X. morstatti* Hag.) et sa plantehote. Dissertationes Wageningen 109 pp.

Bramble, W. C. and E. C. holst. 1940. Fungi associated with *Dendroctonus frontalis* in killing short leaf pines and the effect of conduction. Phytopathology 30: 881-899.

Caird, R. W. 1935. Physiology of pines infested with bark beetles. Bot. Gaz. 96: 709-733.

Callaham, R. Z. and M. Shifrine. 1960. The yeasts associated with bark beetles. Forest Sci. 6: 146-154.

Campbell, R. N. 1958. Nutrient requirements for the production of perithecia by *Ceratocystis variospora* and other species. Amer. J. Bot. 45: 263-270.

Chamberlin, W. J. 1939. The bark and timber beetles of North America, north of Mexico. O. S. C. Co-operative Association, Corvallis, Ore.

Chu, Hsien-Ming, D. M. Norris, and Loke Tuck Kok. 1970. Pupation requirements of the beetle, *Xyleborus ferrugineus*: sterols other than cholesterol. J. Insect Physiol. 16: 1379-1387.

Clemens, W. A. 1916. The pine bark beetle. Cornell Univ. Agr. Expt. Sta. Bull. 383: 287-298.

Cobb, F. W., Jr., M. Krstic, E. Zavarin, and H. W. Barber, Jr. 1968. Inhibitory effects of volatile oleoresin components of *Fomes annosus* and four *Ceratocystis* species. Phytopathology 58: 1327-1335.

Cook, A. H. 1958. The chemistry and biology of yeasts. Academic Press Inc., N. Y. 763 pp.

Craighead, F. C. 1928. Interrelations of tree killing bark beetles *(Dendroctonus)* and blue stains. J. Forest. 26: 886-887.

Davidson, R. W. 1935. Fungi causing stain in logs and lumber in the Southern States including five new species. J. Agr. Res. 50: 789-807.

DeBary, A. 1879. Die erscheinung der symbiose. Karl J. Trubner, Strassburg. 30 p.

Ellis, D. E. 1939. *Ceratostomella ips* associated with *Ips lecontei* in Arizona. Phytopathology 29: 556-557.

Farmer, L. J. 1965. The phloem-yeast complex during infestations of the mountain pine beetle in lodgepole pine. Ph.D. Thesis. University of Utah.

Farris, S. H. 1963. Ambrosia fungus storage in two species of *Gnathotrichus* Eichhoff (Coleoptera: Scolytidae). Can. Entomol. 95: 257-259.

Farris, S. H. and A. Funk. 1965. Repositories of symbiotic fungus in the ambrosia beetle *Platypus wilsoni* Swaine (Coleoptera, Platypodidae). Can. Entomol. 95: 527-536.

Fernando, E. F. W. 1960. Storage and transmission of ambrosia fungus in the adult *Xyleborus fornicatus* Eich. (Coleoptera: Scolytidae). Ann. Mag. Natur. Hist. 13: 475-480.

Finnegan, R. J. 1963. The storage of ambrosia fungus spores by the pitted Ambrosia Beetle, *Corthylus punctatissimus* Zimm. (Coleoptera: Scolytidae). Can. Entomol. 95: 137-139.

Francke-Grosmann, H. 1952. Uber okologie und taxonomie eines mit borkenkafern vergesellschafteten blauepilzes. Proc. 7th Intern. Bot. Congr. Stockholm, 1950 pp. 317-318.

Francke-Grosmann, H. 1956a. Hautdrusen als trager der pilzsymbiose bei ambrosiakafern. Z. Morphol Oekol. Tiere 45: 275-308.

Francke-Grosmann, H. 1956b. Grundlager der symbiose bei pilzzuchtenden holzinsekten. Verhandl. Deut. Zool. Ges. Hamburg (Leipsig) pp. 112-118.

Francke-Grosmann, H. 1958. Uber die ambrosiazucht holzbrutender ipiden im hinblick auf das system. Verhandl. Deut Ges. Angew. Entomol. 14: 139-144.

Francke-Grosmann, H. 1959. Beitrage zur kenntnis der ubertragungsweise von pflanzenkrankheiten durch kafer. Proc. 4th Intern. Congr. Plant Protection, Hamburg, 1957, Vol. I, pp. 805-809.

Francke-Grosmann, H. 1963. Die ubertragung der pilzlora bei dem borkenkafer *Ips acuminatus* Gyll. Z. Angew Entomol. 52: 355-361.

Francke-Grosmann, H. 1965. Ein symbioseorgan bei dem borkenkafer *Dendroctonus frontalis* Zimm. Naturwissenschaften 52: 143.

Francke-Growmann, H. 1966. Symbiosen bei xylomycetophagen und phloeophagen scolytiidea. Material und Organismen 1, Beiheft 1, Symposium, Berlin, Oktober 1965 p. 503-522.

Francke-Grosmann, H. 1967. Ectosymbiosis in wood-inhabiting insects. pp. 141-203. In S. Mark Henry (ed), Symbiosis Vol. 2. Academic Press, N. Y. and London.

Francke-Grosmann, H. and W. Schedl. 1960. Ein orales ubertragungsorgan der nahrpilze bei *Xyleborus mascarensia* Eichh. (Scolytidae). Naturwissenschaften 47: 405.

Franklin, R. T. 1970. Observations on the blue stain-southern pine beetle relationship. J. Georgia Entomol. 5: 53-57.

Giese, R. L. 1966. The bioecology of *Corthylus columbianus* Hopkins. Material und Organism. (In press).

Giodanich, G. 1936. Il genere di ascomyceti *Grosmannia* G. Goid. R. Staz. Pat. Veg. Bol., Rome, n. s. 16: 26-60.

Goeden, R. D. and D. M. Norris. 1965. Some biological and ecological aspects of ovipositional attack in *Carya* spp. by *Scolytus quadrispinosus* (Coleoptera: Scolytidae). Ann. Entomol. Soc. Amer. 58: 771-777.

Gouger, R. J. 1966. A study of artificial feeding and breeding media for the smaller European elm bark beetle, *Scolytus multistriatus* (Marsh.). M.S. Thesis, University of Delaware, Newark.

Graham, K. 1967. Fungal insect mutualism in trees and timber. Annu. Rev. Entomol. 12: 106-126.

Grosmann, H. 1930. Beitrage zur kenntnis der lebens-gemeinschaft zwischen borkenkafern and pilzen. Z. Parasitenk. 36: 56-102.

Hetrick, L. A. 1949. Some overlooked relationships of southern pine beetle. J. Econ. Entomol. 42: 466-469.

Hodges, J. D., S. J. Barras, and J. K. Mauldin. 1968. Amino acids in inner bark of loblolly pine, as affected by the southern pine beetle and associated microorganisms. Can. J. Bot. 46: 1467-1472.

Hogan, T. W. 1948. The biology of the pinhole borer *Platypus subgranosus*. J. Dept. Agr. Victoria 46: 373-380.

Holst, E. C. 1936. *Zygosaccharomyces pini*, a new species of yeast associated with bark beetles in pines. J. Agr. Res. 53: 513-518.

Holst, E. C. 1937. Aseptic rearing of bark beetles. J. Econ. Entomol. 30: 676-677.

Hopping, G. R. 1961. Techniques for rearing *Ips* DeGeer (Coleoptera: Scolytidae). Can. Entomol. 93: 1050-1053.

Hunt, J. 1956. Taxonomy of the genus *Ceratocystis*. Lloydia 19: 1-59.

Jouvenaz, D. P. and R. C. Wilkinson. 1970. Incidence of *Serratia marcescens* in wild *Ips calligraphus* populations in Florida. J. Invert. Pathol. 16: 295-296.

Leach, J. G. 1940. Insect transmission of plant diseases. McGraw-Hill Book Co., N. Y. 615 pp.

Leach, J. G., L. W. Orr, and C. M. Christensen. 1934. The interrelationships of bark beetles and blue-staining fungi in felled Norway pine timber. J. Agr. Res. 49: 315-341.

Lhoste, J. and A. Roche. 1961. Anatomie comparee des organes transporteurs de champignons chez quelques Scolytidae. Proc. 11th Intern. Congr. Entomol., Vienna 1960 I: 385.

Lowe, R. E., R. L. Giese, and M. L. McManus. 1967. Mycetangia of the ambrosia beetle *Monarthrum fasciatum*. J. Invert. Pathol. 9: 451-458.

Lu, K. C., D. G. Allen, and W. B. Bollen. 1957. Association of yeasts with the Douglas-fir beetle. Forest Sci. 3: 336-343.

Mallory, F. B. and J. H. Wright. 1924. Pathological techniques. Eighth edition. W. B. Saunders Company, Philadelphia. 219 pp.

Mathiesen, A. 1950. Uber einige mit borkenkafern associierte blauepilze in Schweden. Oikos 2: 275-308.

Mathiesen, A. 1951. Uber einige neue ophiostoma-arten in Schweden. Svensk Botan. Tidskr. 45: 203-232.

Mathiesen-Kaarik, A. 1953. Eine ubersicht uber die gewohnlichen mit borkenkafern associierten blauepilze in Schweden. Medd. Statens Skogsforskiningsinst. 43: 1-74.

Mathre, D. E. 1964. Pathogenicity of *Ceratocystis ips* and *Ceratocystis minor* to *Pinus ponderosa*. Contrib. Boyce. Thompson Ins. 22: 363-388.

Moreau, C. 1952. Coexistence des formes *Thielaviopsis Graphium* chez une souche de *Ceratocystis major* (van Beyma) nov. comb. Revue de Mycologie, Suppl. Colonial 17: 17-25.

Mrak, E. M. and H. J. Phaff. 1948. Yeasts. Annu. Rev. Microbiol. 2: 1-46.

Nelson, R. M. 1934. Effect of blue stain fungi on southern pines attacked by bark beetles. Phytopathol. 7: 327-353.

Nelson, R. M. and J. A. Beal. 1929. Experiments with blue stain fungi in southern pines. Association of blue stain with bark beetles. Phytopathology. 19: 1101-1106.

Nunberg, M. 1951. Nieco o gruczolach znajdujacych sic w przedtulowiu kornikow (Scolytidae) i wyrynnikow (Platypodidae) (Coleoptera). Ann. Musei. Zool. Polon. 14: 261-265 (contribution to the knowledge of prothoracic glands of Scolytidae and Platypodidae).

Person, H. L. 1931. Theory in explanation of the selection of certain trees by the western pine beetle. J. Forest. 29: 696-699.

Reid, R. W. 1958. The behavior of the mountain pine beetle, *Dendroctonus monticolae* Hopkins, during mating, egg laying, and gallery construction. Can. Entomol. 90: 505-509.

Rennerfelt, E. 1950. Uber den zusammenhang zwischen dem verblauen des holzes und den Insekten Oikos 2: 120-137.

Robinson, R. C. 1962. Blue stain fungi in lodgepole pine (*Pinus Contorta* Dougl. var. *Latifolia* Engelm.) infested by the mountain pine beetle (*Dendroctonus monticolae* Hopk.). Can. J. Bot. 40: 609-614.

Rumbold, C. T. 1931. Two blue-staining fungi associated with bark-beetle infestation of pines. J. Agr. Res. 43: 847-873.

Rumbold, C. T. 1936. Three blue-staining fungi including two new species associated with bark beetles. J. Agr. Res. 52: 419-437.

Rumbold, C. T. 1941. A blue stain fungus. *Ceratostomella montium* n. sp. and some yeasts associated with two species of *Dendroctonus*. J. Agr. Res. 62: 589-601

Schmidberger, J. 1836. Naturgeschichte des apfelborken-kafers *Apate dispar*. Beitr. Obstbaumzucht Naturgesch. Obstbaumen schadlichen Insekten 4: 213-230.

Schneider, I. and J. A. Rudinsky. 1969. Anatomical and histological changes in internal organs of adult *Trypodendron lineatum*, *Gnathotrichus retusus*, and *G. sulcatus* (Coleoptera: Scolytidae). Ann. Entomol. Soc. Amer. 62: 995-1003.

Shifrine, M. and H. J. Phaff. 1956. The association of yeasts with certain bark beetles. Mycologia 48: 41-55.

Siemaszko, W. 1939. Fungi associated with bark beetles in Poland. Planta Polon. 7: 1-54.

Slodki, M. E. 1962. Phosphate linkages from yeast. Biochim. et Biophys. Acta. 57: 525-533.

Slodki, M. E. 1963. Structure of *Hansenula capsulata* NRRL Y-1842 phosphomannan. Brochim. et Biophys. Acta. 58: 96-102.

St. George, R. A. and J. A. Beal. 1927. Progress report on the southern pine beetle (*Dendroctonus frontalis* Zimm.). U. S. Bur. Entomol. Plant Quar. Div. Forest Insect Invest. Ashville, N.C. (Typewritten 40 p.)

Thatcher, R. C. 1960. Bark beetles affecting southern pines: a review of current knowledge. U. S. Dept. Agr., U. S. Forest Serv. South. Forest Exp. Sta. Occasional Paper 180: 35 pp.

Thomas, J. B. 1967. A comparative study of gastric caeca in adult and larval stages of bark beetles (Coleoptera: Scolytidae). Proc. Entomol. Soc. Ont. 97: 71-90.

Vaartaja, O. and P. J. Salisbury. 1965. Mutual effects in vitro of micro-organisms isolated from tree seedlings, nursery soil, and forests. Forest Sci. 11: 160-168.

Vanderzant, E. S. and T. B. Davich. 1956. Laboratory rearing of the boll weevil: a satisfactory larval diet and oviposition studies. J. Econ. Entomol. 51: 288-291.

Verrall, A. F. 1939. Relative importance and seasonal privalence of wood-staining fungi in the southern states. Phytopathology 29: 1031-1051.

Verrall, A. F. 1941. Dissemination of fungi that stain logs and lumber. J. Agr. Res. 63: 549-558.

Wallace, P. 1939. Notes on the smaller European elm bark beetle. Conn. Agr. Exp. Sta. Bull. 439: 293-311.

Webb, S. 1945. Australian ambrosia fungi. Proc. Roy. Soc. Victoria (N. S.) 57: 57-78.

Wickerham, L. J. 1960. *Hansenula holstii*, a new yeast important in the early evolution of the hetero-thallic species. Mycologia 52: 171-183.

Wickerham, L. J. and K. A. Burton. 1961. Phylogeny of phosphomannan-producing yeasts. I. The genera. J. Bacteriol. 82: 265-268.

Wilkinson, R. C. 1962. Stridulating organs in three southeastern *Ips* bark beetles. Fla. Entomol. 45: 43-44.

Wilkinson, R. C. 1964. Attraction and development of *Ips* bark beetle populations in artificially infested pine bolts exposed on firetowers and turntables in Florida. Fla. Entomol. 47: 57-64.

Wilkinson, R. C., W. T. McClelland, Ruth M. Murillo, and E. O. Ostmark. 1967. Stridulation and behavior in two Southeastern *Ips* bark beetles (Coleoptera: Scolytidae). Fla. Entomol. 50: 185-195.

Wilson, C. L. 1959. The Columbian timber beetle and associated fungi in White oak. Forest Sci. 5: 114-127.

Wood, D. L. and J. P. Vité. 1961. Studies on the host selection behavior of *Ips confusus* (LeConte) (Coleoptera: Scolytidae) attacking *Pinus ponderosa*. Contrib. Boyce Thompson Inst. 21: 67-78.

Yearian, W. C. 1966. Relations of the blue stain fungus, *Ceratocystis ips* (Rumbold) C. Moreau to *Ips* bark beetles (Coleoptera: Scolytidae) occurring in Florida. Ph.D. Thesis, University of Florida, Gainesville.

Yu, C. C. and C. H. Tsao. 1967. Gallery construction and sexual behavior in the Southern pine beetle *Dendroctonus frontalis* Zimm. (Coleoptera: Scolytidae). J. Georgia Entomol. Soc. 2: 95-98.

BIOGRAPHICAL SKETCH

Richard James Gouger was born September 24, 1939, at Franklin, New Jersey. He attended elementary and secondary schools in Sussex County, New Jersey, and was graduated from Newton High School in 1958.

He attended the University of Maryland, College Park, Maryland, from September, 1958, until January, 1963, where he received the degree of Bachelor of Science in Agriculture. He entered the Graduate School, University of Delaware, Newark, Delaware, in February, 1963, and was awarded the Master of Science Degree in Entomology in June, 1966.

On February 14, 1968, he married the former Linda Christianne O'Dell of Gainesville, Florida. They have two children, Jennifer Lindsey and Suzanne Elizabeth.

He entered the University of Florida in September, 1965, and was granted a Research Assistantship with the Department of Entomology and Nematology.

In June, 1969, he was appointed to the staff of the Bartlett Tree Research Laboratories, Pineville, North Carolina, as Entomologist.

At present he is a candidate for the Degree of Doctor of Philosophy.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dr. R. C. Wilkinson, Chairman
Professor of Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dr. L. A. Hetrick
Professor of Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dr. James W. Kimbrough
Assistant Professor of Botany

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dr. D. H. Habeck
Associate Professor of Entomoloty and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dr. F. W. Zettler
Assistant Professor of Plant Pathology

This dissertation was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1971

Asst. Dean, College of Agriculture

Dean, Graduate School